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Numerical Density and Diameter of Dentinal Tubules at the Apical Pulpal Wall of Human Mandibular Premolars

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I. INTRODUCTION

Ultrastructure of Dentin

Dentin comprises the majority of tooth structure and serves as both support for overlying enamel and a protective barrier for the pulp. Dentin is composed of oriented tubules surrounded by a highly mineralized peritubular zone, embedded in an intertubular matrix (Marshall 1993). This intertubular matrix consists largely of type I collagen with embedded apatite crystals and dentinal fluid (Marshall 1993). Overall, dentin is composed of about 50% mineral, 30 % organic matrix, and about 20% fluid on a volume basis (Mjör and Fejerskov 1979, Driessens and Verbeeck 1990).

Odontoblasts arise from the dental papilla. Secretory products of the odontoblasts and their processes form the dentin layer (Avery 1984). The odontoblastic processes are extensions of odontoblasts in dentin and are contained within dentinal tubules. Their long cell processes are surrounded by dentin matrix, which is mineralized in mature dentin and unmineralized in immature predentin, so that each process lies within its individual dentinal tubule (Avery 1984). All dentinal tubules run from the dentin-enamel junction (DEJ) to the dental pulp (Linde 1984). It is unclear whether the odontoblastic process in the mature tooth extends all the way through dentinal tubules to the DEJ or only through a small length of the dentinal tubule (Holland 1985, Byers and Sugaya 1995). The extent also varies with regard to location within the tooth (Holland 1976, 1985).

Peritubular dentin is a collagen poor and hypermineralized lining forming within the lumen of the tubule. It is often referred to as " intratubular " dentin (Linde and Goldberg 1993, Pashley 1996). There is a larger amount of intratubular dentin in superficial dentin near the DEJ because it is "older" than middle or deep dentin near the pulpal wall, which is more recently secreted and mineralized (Pashley 1996). The amount of intratubular dentin decreases as the tubules approach the pulp from the periphery. Very close to the pulp, there is no intratubular dentin and the dentinal tubule diameter is at its greatest (Garberoglio and Brännström 1976). Demineralization of dentin samples removes the mineral content of intratubular dentin which can alter the tubule diameters (Arends et al. 1995).

The anatomical diameter of the dentinal tubule decreases as the amount of intratubular dentin increases (Frank and Nalbandian 1989). This intratubular dentin formation continues and may eventually obliterate the tubule. Little is known about the biologic control of intratubular apposition. Presumably, it is a very slow process, slower than the incremental formation of secondary dentin in the pulp chamber (Pashley 1996). The different causes of intratubular dentin formation are argued within the literature. These include age, occlusal attrition, caries, abrasion and response of the odontoblast to external stimuli.

Age and Intratubular Dentin Formation

The most noted possible cause of intratubular dentin formation is increasing age (Nalbandian et al. 1960, Carrigan et al. 1984, Holland 1994). The gradual

increase of intratubular dentin formation with time results in a reduction in size of the dentin tubule (Harcourt 1964, Bradford 1958, Traub et al. 1988). Kvaal et al. (1994) did not find a significant relationship among age and the reduction in diameter size and the number of tubules. "The thickness of intratubular dentin was not so closely related to age that it could be recommended for general use in forensic and archaeological age estimations" (Kvaal et al. 1994). Whittaker (1979) measured tubule diameters near the root apex where age changes were expected to be the most marked, but found no relationship between age and tubule diameter. Mendis and Darling (1979) found a closer relationship between attrition and tubule closure than between age and tubule closure in erupted teeth.

Mjör and Nordahl (1996) used light and scanning electron microscopy to examine the density and branching of dentinal tubules in human teeth. They found no differences in density of tubules between young and old teeth in the coronal , mid-root or apical sections except for the most apical areas of newly formed teeth where root formation was not complete.

Quantifying Dentinal Tubules

Although the primary function of the tubules is to hold odontoblastic processes and tissue fluid, they form important channels to transfer various kinds of stimuli and irritants to the dental pulp. The rate of transfer of such irritants is a function of the tubule density and diameter (Pashley 1987, Marshall 1993). Therefore, it is of interest biologically and clinically to know the number of tubules that are present in a unit area of dentin (Schellenberg et al. 1992).

Pashley (1996) calculated the total area occupied by the lumen of dentinal tubules as the product of the cross-sectional area of a single tubule πr^2 (r=radius of the tubule) and *N*, the number of tubules/ mm^2. Since both the radius of dentinal tubules and their number per unit area increase from the DEJ to the pulp (Mjör and Fejerskov 1979, Fosse et al. 1992, Dourda et al. 1994, Mjör and Nordahl 1996), the area occupied by the tubule lumen also increases (Pashley 1996).

Earlier attempts were made at quantifying human dentinal tubules using a variety of methods including optical and x-ray microscopy. Although the advent of the scanning electron microscope (SEM) offered a more accurate and versatile methodology, inaccuracies still occurred particularly with diameter measurements and tubule counting (Tronstad 1973, Garberoglio and Brännström 1976). This was due to orientation problems of the samples in the SEM and structural changes of dentin samples associated with preparation, especially when using demineralized dentin (Garberoglio and Brännström 1976, Arends et al. 1995, Carvalho et al. 1996).

Use of Mineralized vs. Demineralized Dentin Samples for SEM Analysis

All forms of sample preparation including fixation, dehydration and drying cause significant shrinkage artifacts which can severely limit the quantitative value of scanning electron microscopic studies of the surface morphology when using demineralized dentin (Carvalho et al. 1996). Air drying of sound and especially demineralized dentin causes dimensional changes in the order of 20-30 % (Nyvad et al. 1989, Ruben and Arends 1993a, b). Many of the previous studies failed to address the adverse effects of specimen preparation when using demineralized dentin instead

of mineralized dentin. Arends et al. (1995) studied the diameter of dentinal tubules after in vitro preparation using demineralization and/or air drying, both of which caused dimensional changes prior to examination. They found demineralization of tooth structure increased tubule size initially but then increasing mineral losses eventually decreased the tubule size. The authors believe the mineral crystallites in and on the collagenous structure prevent collagen expansion and if mineral is gradually removed, the collagenous structure expands. They concluded that water losses caused by air drying increase tubule diameter in demineralized dentin. They did not find any change in tubule diameter of the mineralized dentin samples. Kinney et al. (1993), using atomic force microscopy (AFM) to study mineralized dentin, reached a similar conclusion. The initial stages of demineralization were also evaluated with AFM and results indicated that the intertubular matrix collapses during demineralization and alters surface geometry (Marshall 1993).

Garberoglio and Brännström (1976) used scanning electron microscopy to investigate coronal dentin. They used a detailed method for both tubule counting and measurement, and applied it to site-specific areas. They reported 20,000 tubules/mm^2 close to the DEJ and 45,000 tubules/mm^2 close to the pulp. Teeth used in this study were both demineralized and non-demineralized (mineralized). All teeth were dehydrated by freeze-drying. They noted that dehydration caused substantial shrinkage of dentin, especially with demineralized specimens. Demineralization and freeze drying of samples can alter the surface geometry (Marshall 1993). Ten Cate et al. (1991) studied the surface of dried demineralized dentin. They noted that shrinkage

or deformity of dentin occurs and the extent of such shrinkage may be linearly dependent on the volume fraction of mineral content.

Garberoglio and Brännström (1976) recognized that demineralized samples gave false values of tubule diameter and number and attempted to compensate for this by multiplying the measured tubule diameters by an estimated correction factor. The total error in measurement of the diameter of the tubules was approximately 20%. They were the only authors to attempt correction for shrinkage when measuring tubule diameter and number (Pashley 1996).

Drying Methods for SEM Analysis

Drying strongly influences the tubule diameters of demineralized dentin due to water loss and/or shrinkage of the organic matrix (Arends et al. 1995). For sound mineralized dentin, the drying (air and critical point) effects are small or negligible (Arends et al. 1995) and for freeze-dried mineralized dentin, shrinkage is only 5% in volume (Van der Graff and Ten Bosch 1990).

The most widely recommended drying method for preparation of demineralized and mineralized dentin specimens for the SEM is critical point drying. A number of disadvantages exist, however, with this technique including preparation time required, specific equipment needed and surface cracking of specimens after dehydration (Carvalho 1996). Hexamethyldisilazane (HMDS) is an alternative method that produces a similar amount of specimen shrinkage when compared to critical point drying (Carvalho 1996). The mechanism of action of HMDS is not fully understood.

The association of low surface tension and cross-linking potential is likely to be an important factor (Bray et al. 1993). HMDS provides better dimensional preservation for fixed specimens (Carvalho 1996). It also preserves the collagen network better and the microporosity of the demineralized dentin surface (Perdigao et al. 1995) which is important when investigating dentin microstructure. There is also a significant decrease in surface cracking and thickening of the denatured collagen layer often seen with air and critical point drying (Perdigao et al. 1995). The main advantages of HMDS over critical point drying are no costly equipment required, many samples can be prepared at once and minimal time is required for specimens to dry (Bray et al. 1993). HMDS is also miscible with alcohol and acetone (Heegaard et al. 1986), both of which are used for dehydration of dentin specimens.

Stage Tilt During SEM Analysis

When viewing dentin samples in the SEM, it is very difficult to obtain a straight on overview of all dentinal tubules in a given field, regardless of how the specimen is oriented on the SEM stage or the SEM stage is tilted in relation to the electron beam. This is due to their anatomical location and differing orientations within a given field (e.g. Figs. 1, 2, 3, 4). This was not taken into account in most of the previous studies in the literature. Only two studies took this into consideration. Garberoglio and Brännström (1976) positioned the area of the specimen to be studied as perpendicular as possible to the electron beam. This was accomplished by using a large SEM aperture (200 μm) which resulted in a small depth of focus. The investigators then determined that by checking the depth of focus on the micrographs,

the tilt angles were kept smaller than 30°. The error in measuring the diameter caused by the specimen tilt was estimated to be less than 15%. They calibrated the magnification of the SEM by filters and standard size spheres. The magnification was estimated to be within 10% of the correct value. When counting the number of tubules, their combined errors in magnification and tilt angle were approximately 25% (Garberoglio and Brännström 1976).

Arends et al. (1995) used a computer program to measure tubule diameter nearly independent of the tubule orientation. "The procedure used measures the smallest inner diameter of a tubule, which makes the varying tubule direction unimportant for the final result " (Arends et al. 1995). Information regarding details of this method were lacking in the publication however.

Quantifying Dentinal Tubules; Tooth and Site Specificity

Anatomy-dependent regional differences regarding tubule density and size were not recognized by earlier investigators who generally sampled from unspecified teeth and non-specific sites. This led to a wide range in reported tubule density. Scanning electron microscopy (SEM) coupled with morphometric analysis were used recently to analyze the percentage of dentinal surface area occupied by dentinal tubules (percentage area) at various levels (Dourda et al. 1994). In this study, fractured mineralized sections of coronal dentin were examined. In coronal dentin near the DEJ, the number of tubules was reported to be 22,000/mm^2 and percentage area was 3.6%, and close to the pulp the number of tubules was 48,000/mm^2 and the percentage area of tubules was 10.2% (Dourda et al. 1994). "The number of tubules per square

millimeter more than doubled and the area occupied by these tubules increased three-fold from the dentin close to the DEJ to that close to the pulp " (Dourda et al. 1994).

Tronstad (1973) studied coronal dentin with scanning and transmission electron microscopy. He reported the number of dentinal tubules in mineralized dentin to be 7000 tubules/mm^2 close to the DEJ and 60,000 tubules/mm^2 close to the pulp. According to the study, the diameter of tubules near the DEJ were much smaller than those close to the pulp (Tronstad 1973). These figures however, were reported without describing a systematic investigation method for data collection.

There are only a few studies addressing the numerical tubule density appearing near the inner wall of the pulp chamber (Tronstad 1973, Garberoglio and Brännström 1976, Whittaker 1979, Mjör and Nordahl 1996) . In most of the studies, however, tubules were counted in either unspecified teeth and/or non-specific sites. This may account for the wide range in tubular densities reported and cannot be considered as representative for a given dento-anatomical site. Schellenberg et al. (1992) obtained site specific data of tubule density of specified human teeth at the pulpal wall of permanent teeth in the coronal and mid-root levels. They found the number of tubules present on the buccal/lingual walls was significantly higher than those on the mesial/distal walls and tubule density of the pulpal aspect of the coronal dentinal wall was significantly greater than that of the radicular wall. Therefore, in expressing dentinal tubule density, it is important to relate the data to the tooth and to the specific dento-anatomical region from which they are collected (Schellenberg et al. 1992).

Using a new method, Fosse et al. (1992) studied the numerical density, and distributional pattern of transversely cut dentinal tubules as well as the diameters of their intratubular dentin walls. These values were measured in sections of coronal dentin near the DEJ, midway to the pulp, and near the pulpal wall in human premolars. At all three levels the measurements comprised the same bundle of tubules from the DEJ to the pulp. The number of tubules per square millimeter increased more than three times from the DEJ to the pulpal wall. A pulpward decrease in the thickness of peritubular dentin was also quantified.

Although coronal dentin has been studied extensively (Tronstad 1973, Brännström and Garberoglio 1976, Dourda et al. 1994), there is limited literature dealing with dentin in the apical one-third of teeth (Tidmarsh and Arrowsmith 1989, Fosse et al. 1992, Mjör and Nordahl 1996). In one study (Tidmarsh and Arrowsmith 1989), at a point approximately 3mm from the apex, the tubule density was recorded in three locations: close to the pulpal wall (28,000 tubules/mm^2), halfway between the pulp canal and dentin-cementum junction (18,000 tubules/mm^2), and just inside the dentin-cementum junction (13,000 tubules/mm^2). However, data on dentinal tubule density at the pulpal wall were not included in this study.

Garberoglio (1994) studied the angle formed by the dentinal tubules with a hypothetical class II cavity preparation in human teeth. Results of this study indicated the density of dentinal tubules on the cervical wall in coronal dentin was higher than that on the axial wall. Recently, Mjör and Nordahl (1996) determined that the number

of branches of dentinal tubules was more abundant in locations where the density of tubules was low such as in the root and in coronal and mid root peripheral dentin.

Dentin Permeability

Dentin can be regarded as "a porous biologic composite made up of apatite crystal filler particles in a collagen matrix" (Pashley 1996). Dentin is permeable because millions of microscopic fluid-filled tubules penetrate the mineralized matrix and extend from the pulpal wall to the periphery of the tooth surface. Pashley (1996) has shown that dentinal tubules are major channels for solute diffusion and movement of fluid across dentin between periphery and pulp. This movement can occur in both directions. Exogenous materials such as microbes, their by-products and their toxins can diffuse across dentin (Pissiotis and Spångberg 1992). A small positive pulpal pressure (Beveridge and Brown 1965, Vongsavan and Matthews 1992) causes an outward flow of dentinal fluid (Brännström and Åström 1972) which may serve as a protective barrier against the inward flux of potentially irritating exogenous materials (Pashley 1996).

Pashley et al. (1987) have shown that dentinal permeability is highly variable and is dependent on location within the tooth. Permeability is greater near the pulp than towards the periphery. This is a consequence of the increase in dentinal tubule density (number of tubules per mm^2) , the increase in diameter of each dentinal tubule and the convergence of tubules as they approach the pulpal wall (Tronstad 1973,

Garberolgio and Brännström 1976, Pashley 1991, Fosse 1992, Mjör and Nordahl 1996).

Most substances move across dentin by simple diffusion. Variations in permeability may arise from regional differences, tubular irregularities associated with mineral deposits and organic components of the odontoblastic process (Marshall 1993). The permeability of the odontoblastic layer has been shown to increase under severe dentinal irritation (Turner 1992). Intratubular deposits of collagen (Dai et al. 1991), the thickness of remaining dentin and the presence of a smear layer can also affect permeation rate of solutes (Pashley 1996).

Clinical Considerations

Pulpal and periapical pathosis is caused by exposure of the root canal system to microorganisms, their by-products and their toxins (Kakehashi et al. 1965, Bergenholz 1974, Sundqvist 1976, Möller et al. 1981, Fabricius et al. 1982). This exposure may occur via presence of caries (Reeves and Stanley 1966, Brännström and Lind 1965), enamel and dentin cracks (Walton et al. 1984, Bergenholz 1974, Ehrmann 1990), infection of exposed dentinal tubules (Sundqvist 1976, Sen et al. 1995) and accessory canals (Gutmann 1978), coronal microleakage (Cox and Bergenholz 1985, Walton 1987, Madison and Wilcox 1988), traumatic injuries (Sundqvist 1976) and possibly systemic circulation routes (Gier and Mitchell 1968). Endodontic pathogens may also aggravate "marginal infection" in areas of the root devoid of cementum (Ehnevid 1995).

Conventional endodontic therapy involves elimination of microorganisms and their by-products from the root canal system with proper cleaning and shaping using a variety of chemical and mechanical techniques. This is followed by complete obturation of this system to create an effective barrier to prevent further passage of microorganisms or their products to the surrounding periapical and radicular tissues.

Success rates for conventional endodontic therapy have been reported as low as 45% to as high as 98% (Torabinejad and Pitt Ford 1996). Major factors affecting prognosis include residual infection (Engström et al. 1964, 1965), presence and size of apical periodontitis (Strindberg 1956), overinstrumentation of the apical foramen and presence of excess filling material (Bergenholz et al. 1979). Several factors contribute to long term success including proper cleaning, shaping, and obturation (Dow and Ingle 1955), sufficient apical seal (Weine 1982), adequate coronal seal with a well fitting permanent restoration (Buckley and Spångberg 1995, Ray and Trope 1995) and the time elapsed between completion of endodontic therapy and placement of permanent restoration (Safavi et al. 1987, Torabinejad et al. 1990).

Conventional endodontic therapy failures may occur due to residual bacteria and their products in the pulp space. Dentinal tubules of the root canal walls have been shown to harbor microorganisms (Haapasalo and Ørstavik 1987, Safavi et al. 1990, Sen et al. 1995). If endodontic therapy fails, retreatment is indicated and often results in treatment success (Bergenholz 1979).

Several clinical conditions are encountered during conventional treatment in which non-surgical endodontic therapy is unable to meet the proper objectives. These include excessive calcification of the root canal system, shoulders or ledges, strip perforations of the canal, teeth recently restored with post and core crowns, and cases which remain symptomatic after conventional treatment (King et al. 1990). Other factors include complex anatomy and physical barriers such as separated instruments and unretrievable cores and silver points.

Surgical endodontic therapy is then required and often involves removal of an infected or damaged root apex and placement of a retrograde filling. The retrograde filling is placed to prevent penetration of tissue fluids into the root canal system or leakage of microorganisms and their toxins through the apical foramen into the surrounding tissues. Inefficient retrograde sealing of the root canal following root-end resection has been suggested as being a major factor in surgical endodontic failure (Rud et al. 1972).

The leakage of root-end fillings has been the subject of numerous investigations in the past (Friedman 1991, Torabinejad and Pitt Ford 1996). An ideal material to seal root-end cavities should be non-toxic, non-carcinogenic and biocompatible with the host tissues. It should prevent leakage of microorganisms and their by-products into periradicular tissues. In addition, it should be insoluble in tissue fluids and dimensionally stable. The presence of moisture should not affect its

sealing ability. The material should be easy to use and be radioopaque (Gartner and Dorn 1992).

A number of materials have been evaluated for use as root-end filling materials. These include amalgam, gutta percha, polycarboxylate cements, Intermediate Restorative Material, Super Ethoxy Benzoic Acid cement, composite resins, glass ionomers, Cavit, gold foil and leaf, silver points, cyanoacrylates, poly HEMA and Hydron and in recent years Mineral Trioxide Aggregate (Torabinejad and Pitt Ford 1996). The suitability of root-end filling materials has been tested for physical properties such as leakage and marginal adaptation, and for biologic properties through cytotoxicity tests and implantation assays. Finally, they have been the subject of usage tests in experimental animals and man (Torabinejad and Pitt Ford 1996). The reports regarding the prognosis of apical surgery in relation to retrograde filling of the root canal are inconsistent (Friedman 1991).

The objective of root-end preparation and filling is to establish an adequate apical seal between the root canal system and the surrounding periapical tissues. It has been suggested that leakage can occur along the interface between the filling material and the canal wall (Guttman and Pitt Ford 1993, Torabinejad and Pitt Ford 1996). It has also been speculated that leakage can occur by flow of fluids and microorganisms through open dentinal tubules at the resected root-end (Gilheany et al. 1994). Gilheany et al. (1994) described the sum of these two pathways as "apical leakage".

Apical Dentin Permeability

The role of permeable apical dentin is poorly understood (Gilheany et al. 1994, Tidmarsh and Arrowsmith 1989). Few studies have examined the influence of the root-end preparation/apical bevel design and its relationship to apical dentin permeability (Weine 1982, Arens 1981, Beatty 1986). Beatty (1986) demonstrated that the seal of the root-end filling is compromised if the cavity preparation is not extended coronally into the root canal to at least the height of the bevel (Fig. 5a). Extending the cavity preparation and placing the root-end filling to this level were found to restrict apical fluid penetration into the root canal space through exposed dentinal tubules (Fig. 5b) (Beatty 1986, Gilheany et al. 1994). Steeply angled root-end resections are reported to allow more leakage between the cut face of the resected root surface and the pulpal wall of the root canal (Vertucci and Beatty 1986). Tidmarsh and Arrowsmith (1989) used scanning electron microscopy to examine the resected root-ends of teeth. They concluded that the angle of the bevel should be kept to a minimum and that the retrograde filling should extend coronally into the canal, at least to the level of the coronal end of the bevel. This would minimize the number of dentinal tubules communicating between the resected root surface and the pulpal wall. Gilheany et al. (1994) reported that as the root end bevel angle is increased, dentin permeability and microleakage increases. They also reported that as the depth of the root-end preparation and retrofilling is increased the dentin permeability and microleakage decreases.

Permeability is site-specific (Pashley 1987) and the importance of exact location of tubules should be noted as there are significant differences between apical vs. coronal dentin as well as dentin close to the DEJ vs. dentin close to the pulpal wall (Mjör and Nordahl 1996). Apical dentin permeability may be an important factor affecting the prognosis of periapical surgery, especially the influence of exposed dentinal tubules created during root-end preparation. The permeation of substances such as microbial irritants across dentin are related to the density of dentinal tubules (# tubules/mm^2) and their diameters (Pashley and Pashley 1991). The percentage area of dentin occupied by dentinal tubules is a function of both the number of tubules and the individual tubule diameter. The rate of diffusion of materials through dentin is directly proportional to the percentage area of the dentinal tubules (Pashley 1988). The percentage area of dentinal tubules in a given area of dentin increases significantly as the pulpal wall is approached from the periphery (Dourda et al. 1994). The density of dentinal tubules at the pulpal wall in the apical 6 millimeters of human root canals is not known. The purpose of this study is to determine the numerical density, diameter, and percentage area of dentinal tubules at the pulpal wall in the apical 6 mm of human permanent mandibular premolars.

II. SIGNIFICANCE

The major goal of root-end preparation and filling is to prevent communication between the root canal system and periapical tissues. There are potentially two avenues by which microleakage can occur after root-end preparation and filling: 1) the area of exposed dentinal tubules of the prepared root apex and 2) root-end filling material /dentin interface. Reports on microleakage associated with filling material/dentin interface abound in the literature. Microleakage associated with exposed dentinal tubules, on the other hand, has been the subject of very few studies and its impact on periapical surgery prognosis has not been investigated.

Current clinical recommendations regarding root-end filling procedures are based, to a large extent, on the results of in vitro dye leakage tests in coronal dentin. Based on the results of these studies, one may assume that in apical dentin, the rate of transfer of irritants is a function of dentinal tubule density, tubule diameter and percentage area occupied by the tubules. Information on apical tubular density, therefore may have a significant value for the discussion of endodontic surgical techniques.

III. MATERIALS AND METHODS

Two pilot studies (I and II) were completed to determine the proper preparation techniques required for sample evaluation in the SEM. A third pilot study (III) was performed to verify the accuracy of methods used for data collection and analysis.

Pilot Study I

A total of eight intact, sound human teeth were used for pilot studies (I) and (II). The teeth consisted of permanent mandibular premolars only, extracted from an adolescent population. Upon extraction, the teeth were fixed in 2.5% glutaraldehyde in 0.1 *M* sodium cacodylate buffer (pH=7.4) for four to five days and then stored in 0.1 *M* cacodylate buffer.

Pilot study (I) was completed to determine the proper tooth sectioning method required for SEM sample preparation. Eight teeth were divided into two groups (A and B) to evaluate two different sectioning methods. The crowns of teeth in both groups were removed at the level of the cemento-enamel junction (CEJ) to facilitate proper mounting of the root segments for SEM evaluation.

In group A, a total of four teeth were used. Each tooth was mounted in a plastic container surrounded by sticky wax. This allowed for proper placement of the tooth onto the mounting jig. Each tooth was sectioned along the buccal/lingual plane into two segments with a 300μm diamond disc saw (Buehler Isomet Low Speed Saw)

with a distilled water reservoir. Due to the straight cutting plane of the blade, teeth with even the most minimally curved roots could not be mounted and sectioned properly (Fig. 6). Also, certain teeth had a very narrow apical portion and these teeth could not be negotiated. Therefore, only teeth with straight roots were used with this technique.

In group B, a total of four teeth were used. The teeth were sectioned by creating a 1-2 mm groove into dentin along the periphery of the long axis with a high speed handpiece and #169 carbide bur. The teeth were then sectioned along the buccal/lingual plane into two segments using a mallet and chisel (Fig.7). A total of eight tooth segments were prepared in group B. It was possible to section teeth with straight or minimally curved roots. Teeth with very narrow apical portions were often effectively negotiated and sectioned with this technique.

Pilot Study (II)

Pilot study (II) was completed to determine the proper dehydration/drying method of the teeth segments required for SEM analysis. Four different dehydration/drying methods were tested. The eight teeth segments sectioned by the mallet/chisel method were used as samples in pilot study (II). The prepared segments were transferred to 5.25% NaOCl ($pH \cong 12$) and placed on an automatic rocker machine at room temperature to dissolve residual tissues. The 5.25 %NaOCl solution was replaced with fresh solution each half hour for a total of 3 hours. The segments were then rinsed with distilled water 3 times and transferred to an ultrasonic unit to

dislodge any remaining debris. The segments were then examined under a stereo microscope at 24X to confirm complete removal of soft tissue along the pulpal wall and then rinsed with distilled water.

The eight tooth segments were divided into four groups (I, II, III, and IV). In group I, two tooth segments were air dried in a desiccator for a minimum of 24 hours. For group II, two tooth segments were dehydrated in ascending grades of ethanol (25%, 50%, 75%, 95%) for one hour in each concentration. The segments were transferred to 100% ethanol for two applications of one hour each and then stored in the desiccator. Group III consisted of two tooth segments dehydrated with ascending grades of ethanol and then subjected to critical point drying (BOMAR-SPC900EX/transitional medium=liquid CO_2). They were then stored in the desiccator. The final group (IV) consisted of two segments dehydrated in the same ascending ethanol series and then placed in HMDS solution for 30 minutes. This group was also transferred to the desiccator for storage.

The tooth segments from each group were then mounted on aluminum stubs. Carbon tape was placed over the aluminum discs and the tooth segments were attached to the tape. The tooth segments were supported with lead foil from dental film packets so that the long axis/pulpal wall was parallel to the aluminum stub for accurate viewing with the SEM. Conductive carbon glue was then applied to areas of the tooth in contact with the lead foil or aluminum stub to prevent charging of the specimens

during SEM analysis. The samples were then sputter coated (Hummer X- Anatech Ltd.) with gold (15 *n*m) and were ready for viewing with the SEM (JEOL JSM-35CF).

Main Study

A total of 20 healthy, caries-free human permanent mandibular premolar teeth were used as samples. The teeth were extracted from teenage children for orthodontic reasons. Upon extraction, the teeth were fixed in 2.5% glutaraldehyde in 0.1 *M* sodium cacodylate buffer (pH = 7.4) for four to five days and then stored in 0.1 *M* sodium cacodylate buffer.

The teeth were sectioned using the mallet/chisel technique and dehydrated/ dried with the ethanol/HMDS technique. These samples were then prepared for SEM analysis as previously described and stored in a desiccator.

A method for accurately measuring tubule diameter was developed and used in this study. Individual samples were placed onto the SEM stage and the stage loaded into the SEM. Standardized SEM micrographs were recorded and compared to previous studies (Schellenberg et al. 1992, Fosse et al. 1992) to determine standardized inspection sites and magnification necessary for accurate measurement of tubule areas. Magnification settings of 10X, 100X and 1000X were selected (Figs 8, 9, 10). A standardized grid was created and superimposed onto the SEM monitor at 10X. This grid allowed for accurately locating the apical 1, 3 and 6 mm where data were to be collected. This was later modified by also notching the tooth segments, in cementum at these levels, prior to dehydration, to confirm accurate measurement

locations. A second standardized grid was created to make a standardized random method for selecting tubules at 1000X. This grid was a modified version of Gutierrez method (Gutierrez et al. 1990) for scanning dentinal tubules and was applied to the apical 1, 3 and 6 mm level of each tooth segment. At each level, the center of the pulpal wall of the root canal at 1000X was labeled site #1 and an SEM micrograph was taken. From site #1, the tooth segment was moved on the SEM specimen stage 0.05 mm laterally to the left, then 0.05 mm vertically downward to site #2 which was to the left side of the root canal wall and apical to site #1. A micrograph was then taken at site #2. Site #3 was located 0.05 mm laterally to the right of site #1 and 0.05 mm vertically upward from site #1. This examined the right side of the root canal wall and was coronal to site #1. A micrograph was then taken at site #3. The standardized grid created a standardized random method (sites #1, 2 and 3) for scanning the root canal walls horizontally and vertically at each of the apical 1, 3, and 6 mm levels for each tooth segment. Therefore, at 1000X, each tooth segment was viewed at levels 1, 3 and 6 mm from the apex (Figs.11,12, 13) At each level, 3 micrographs were taken using the standardized random method. A total of 9 micrographs per tooth segment were recorded resulting in a total of 360 micrographs for the study.

The micrographs were labeled and digitized with a flatbed scanner (Bochacki et al. 1992) and transferred to an optical disc for storage. A Silicon Graphics Imaging/Onyx super-graphics computer system (Unix operating system) was used with Adobe Photoshop software to analyze the images.

Adobe Photoshop allowed for automated selection and measurements of the dentinal tubules (1000X) by recognition and comparison of gray scale shades. The dentinal tubules were very dark shades (Fig.14) of gray (black) and thus easily selected out by the program. However, with this automated selection process, there was a significant amount of artifact (Fig.14) also selected as tubules by the program. This was corrected by using the Adobe Photoshop program tools to manually select each tubule in the image. Each image was divided into four segments on the computer screen. Each area could be magnified and the circumference of the tubule could be selected by viewing the individual pixels and change in pixel shade. This manual method for tubule selection, although time consuming, proved to be very accurate and ruled out unwanted artifact.

The image containing dark circular areas (tubules) was then inverted to create white circular areas on a black background for easier site inspection and recognition (Fig.15). The inverted image was labeled and referred to as *Mask 1* (the computer program default term) for that original image (i.e. *Mask 1* of micrograph #X) and saved in this format on an optical disk.

Mask 1 was imported and analyzed with Cantata Visual Programming Language for Khoros Image Processing software (Cantata/Khoros). This program inspects the imported *Mask 1* image and counts the number of tubules in the given field (# tubules/image) by recognition and comparison of pixel shades of black or white. There were no other pixel shades in the *Mask 1* image for the program to

recognize and thus automated counting by the program was completed without any artifact. These values (# tubules/image) were recorded with the corresponding micrograph and stored for calculation of the number of tubules per square millimeter (# tubules/mm^2) later in the study.

A method was developed to compensate for the anatomical and morphological orientation problems associated with the measurement of tubule diameter and percentage area of tubules. The original SEM image and its inverted image of selected tubules, *Mask 1*, were examined and then used to select those tubules which were viewed in a straight on orientation. Tubules which were minimally distorted and intact circumferentially were selected. Approximately 20-25 tubule diameters were measured in each *Mask 2* image. This ruled out tubules visually distorted by sample orientation with respect to the electron beam of the SEM and minimized observer bias.. These tubules were then manually selected in Adobe Photoshop as previously described for *Mask 1* images and then saved as *Mask 2* (Fig. 16). *Mask 2* was then imported into the Cantata/Khoros program and used to calculate tubule diameter in pixels and combined with *Mask 1* to calculate percentage area occupied by the tubules. *Mask* 1 was used to calculate the number of tubules per square millimeter (tubules/mm^2).

The Cantata/Khoros program converts pixels to microns for measuring the number of tubules per square millimeter (tubules/mm^2), tubule diameter in micrometers (μm), and calculates the percentage area (% area) occupied by tubules.

In order to compute these values for the 360 micrographs, the program required two calculations : 1) Mean value (from 360 micrographs) of the number of micrometers per pixel (#µm/pixel) in a given image, (2) Mean individual micrograph area (μm^2) representing the 360 micrographs taken.

To calculate these values, the dimensions (height and width) of the 360 micrograph images were measured in pixel units and the mean dimensions were calculated. Each SEM micrograph image contains a measurement bar (µm) at a given magnification. The measurement bar at 1000X represented 10µm. By measuring this bar in pixels, the number of micrometers per pixel (µm/pixel) was determined. This was used as a constant value to convert the height and width measurements to micrometers (µm), and then the area of the image (μm^2).The mean values of micrometers per pixel (µm/pixel) and area (μm^2) of the 360 images were then recorded and used as constant values for data analysis of the tubules at each level.

To summarize:

Using mean values from 360 SEM micrographs;

(1) **Calculation of µm/pixel**

Mean size of 10 µm bar for 360 micrographs = 105 pixels

# of µm/pixel = 10 µm ÷ 105 pixels

Calculation of µm/pixel = 0.095 µm/pixel (constant)

(2) **Calculation of Area (μm^2)**

Mean Height of 360 Micrographs = 922 pixels × 0.095 μm/pixel = 87.59μm^2

Mean Width of 360 Micrographs = 1184 pixels × 0.095 μm/pixel = 112.48μm^2

Mean Area of all micrograph images = 87.59 μm × 112.48 μm

Calculation of Area (μm^2) = 9852.12 μm^2 (constant)

The Cantata/Khoros program then applies these constant values to the data measured from each micrograph (# tubules/image counted from *Mask 1* and average tubule diameter in a given micrograph calculated from *Mask 2*) to calculate the number of tubules per square millimeter (tubules/mm^2), tubule diameter in micrometers (μm), and the percentage area occupied by tubules (% area).

By using data from *Masks 1* and *2*, Cantata/Khoros program performs the following calculations for each micrograph:

Tubules/mm^2

(1) 9852.12 μm^2 (constant) **x** (1mm^2 ÷ 1,000,000 μm^2)

= 0.010 mm^2(constant)
Mean area of all micrographs (mm^2)

(2) Number of tubules in sample micrograph *(Mask 1)* ÷ 0.010 mm^2(constant)
= **Tubules/mm^2**
of sample micrograph

Tubule Diameter (μm)

Average pixel diameter (*Mask 2*) **x** 0.095 μm/pixel (constant)
of sample micrograph

= **Tubule Diameter (μm)** of sample micrograph

% Area

$$\left[\begin{array}{l}\text{number of tubules } \textit{(Mask 1)} \\ \text{in sample micrograph}\end{array}\right] \mathbf{X} \left[\begin{array}{l}\pi \text{ (Tubule Diameter } \mu m \div 2)^2 \\ \text{in sample micrograph}\end{array}\right] \div 9852.12 \mu m^2 \mathbf{X}\ 100$$

= % Area tubules occupy

The average numerical tubule density (tubules/mm^2), average tubule diameter (μm), and percentage area occupied by dentinal tubules (% area) were calculated at specific sites on the pulpal wall at the apical 1 mm , 3 mm and 6 mm levels. These data were collected for 360 micrographs and stored in the computer software program Microsoft Excel and transferred to Statistical Program for Social Sciences (SPSS for MS WINDOWS) software program for statistical analysis. Means of all outcome measures were compared across the three apical levels using a one-way analysis of variance. Multiple comparison procedures were used to isolate paired mean differences where appropriate.

Pilot study (III)

The accuracy of the data collection and analysis method was tested in pilot study (III) which was performed at the beginning of the study. A total of seven tooth segments were sectioned and prepared for SEM analysis. A total of three groups were used (A, B, and C). In group A, the stage tilt was set at 0° which oriented the tooth segment perpendicular to the electron beam of the SEM. The tilt for group B was set

at +4° and group C tilt set at -4°. After sampling different tilt settings, the +/- 4° tilt setting was selected by the operator to most closely simulate the anatomical and morphological orientation problems associated with tubule measurement. The three groups examined a given micrograph location at three different tilt settings.

Each group (A, B, and C) contained one micrograph taken of a specific location at each of the three apical levels (1 mm, 3 mm, and 6 mm). This was done with each of the seven tooth segments. Therefore, group A consisted of 21 micrographs taken with a stage tilt setting of 0°. Groups B and C each consisted of 21 micrographs also, at stage tilt settings of +4° and -4° tilt respectively. The data for the 63 micrographs were then collected and analyzed with the method previously described. The accuracy of the selection method was tested using oneway analysis of variance and Levene test for homogeneity of variances. Data were compared between the three tilt settings at a given location.

IV. RESULTS

Results of pilot study (III) were completed at 21 different locations and indicated a low coefficient of variation (0.01-0.06) when comparing the three different tilt settings at a given location (Table 1).

Results of the main study regarding average numerical tubule density (tubules/ mm^2), tubule diameter (μm) and the percentage area occupied by dentinal tubules (% area) were recorded in Tables (2-4) and Figures (20-23).

The average numerical tubule density (tubules/mm^2) was found to be significantly less ($p < 0.0001$) at the apical 1 mm level (17,067 tubules/mm^2) compared to the apical 6 mm (48,440 tubules/mm^2) (Table 2, Figs. 20, 23). The apical 3 mm level (36,623 tubules/mm^2) was also significantly less ($p \leq 0.0001$) than the apical 6 mm level. One-way analysis of variance indicated significant differences among means across the three levels ($p \leq 0.0001$) of numerical tubule density. The coefficient of variation for each level was ≤ 0.13. The greatest degree of variation was seen at the 6 mm level where the 95% confidence interval for the mean was (47352, 49527). The minimum value recorded at the apical 6 mm level was 38,516 tubules/mm^2 and the maximum value recorded was 69, 533 tubules/mm^2.

One-way analysis of variance indicated significant differences among means across each of the three levels ($p \leq 0.0001$) of percentage area occupied by dentinal tubules (% area) (Table 3, Figs. 21, 23). Dentinal tubules occupied 2.95% of the area

at the apical 1 mm level, 7.27% at the apical 3 mm level and 11.57% at the apical 6 mm level.

Regarding tubule diameter (μm), one-way analysis of variance indicated significant differences among means across each of the three levels ($p \leq 0.0001$) (Table 4, Figs. 22, 23). The tubule diameter means varied from 1.10 (μm) at the apical 1 mm level to 1.52 (μm) at the apical 6 mm level. The highest standard deviation was noted at the apical 1 mm level (SD=0.24). The minimum value recorded (0.80μm) was quite different from the maximum value (2.01μm). However, the coefficient of variation at the apical 1mm level was ≥ 0.22.

The average numerical tubule density, diameter and percentage area varied significantly $p \leq 0.0001$ among the three levels. The range of the results within each group was moderate. When comparing the apical 6 mm to the apical 1 mm, the number of dentinal tubules nearly tripled and the area occupied by these tubules increased more than four-fold (Fig.23). The diameter of the tubules increased in size by approximately 40%. Comparing the apical 3 mm level to the apical 1mm level, the number of dentinal tubules doubled and the percentage area increased nearly three-fold. Tubule diameter increased approximately 16%.

V. DISCUSSION

The primary objective of this study was to obtain tooth and site-specific data on the numerical density, diameter and percentage area occupied by dentinal tubules in the apical third of human root canals. A total of 20 mandibular premolars from an adolescent population was studied. The average numerical tubule density (tubules/mm^2), average tubule diameter (μm), and percentage area occupied by dentinal tubules (% area) calculated at the apical 1mm, 3 mm and 6 mm levels were: 17,067 tubules/mm^2, 1.10μm, 2.95%; 36,623 tubules/mm^2, 1.28μm, 7.27%; and 48,440 tubules/mm^2, 1.52 μm, 11.57%; respectively. This is consistent with previous findings by Tidmarsh (1989) where measurements were taken close to the pulpal wall 3mm from the apex with a range of 19,000 - 48,000 tubules/mm^2 with a mean of 28,000 tubules/mm^2 (SD 8410). The results were also consistent with the overall trend of dentinal tubule density increasing from the radicular wall to the coronal dentinal wall Schellenberg (1992) as the the number of dentinal tubules nearly tripled and the area occupied by these tubules increased more than four-fold (Fig.23)when comparing the apical 6 mm to the apical 1 mm .

There are a limited number of studies addressing the numerical tubule density near the inner wall of the pulp chamber (Tronstad 1973, Whittaker 1979, Schellenberg 1992, Tidmarsh and Arrowsmith 1992, Dourda 1996 Mjör and Nordahl 1996). The majority of these studies compare tubule density at the DEJ to that of the

pulpal wall. The density of dentinal tubules at the pulpal wall in the apical 6 millimeters of human root canals is not known. This study established values for average numerical tubule density, diameter and percentage area 1mm, 3mm and 6mm from the anatomical apical foramen. The average numerical tubule density, diameter and percentage area varied significantly $p \leq 0.0001$ among the three levels. One-way analysis of variance indicated significant differences among the means across each of the three levels.

Undemineralized (mineralized) dentin was used in this study as there is evidence that intratubular dentin is soluble to a large extent during demineralization (Brännström and Garberoglio 1972). The effects of demineralization on surface morphology and dimensional changes have also been reported (Ten Cate 1991, Arends et al., 1995, Carvalho 1996). During SEM analysis, demineralized dentin specimens undergo volumetric shrinkage of 10-20 % when measured in the vacuum chamber of the SEM (Carvalho 1996). Specimen preparation effects are small or negligible in sound mineralized dentin (Arends et al. 1995). Only mineralized dentin was used in this study.

The results of group A from the first pilot study indicated the manual sectioning of teeth with a mallet and chisel was the preferred method. The diamond disc saw method did not allow for adequate sectioning of even slightly curved roots due to the straight cutting plane of the blade along the curved long axis of the root. It was also difficult negotiating the diamond disc saw along the narrow apical portion

of the teeth. Mounting these teeth for sectioning was also difficult. Due to these difficulties experienced with this technique, several teeth had to be discarded. Using the mallet and chisel sectioning technique, teeth with straight and minimally curved roots were sectioned consistently. It was possible to negotiate and section the narrow apical portion of these teeth. There was also no mounting of specimens involved as in the other technique. The manual sectioning of teeth with the mallet and chisel consistently produced the best samples and was the preferred method.

In group B of the first pilot study, the preferred dehydration/ drying method was using the ethanol/HMDS series. Air and ethanol/critical point drying both produced significant surface cracking of the samples (Figs.17,18) and thus made analysis difficult. This surface cracking increased with time during storage as well (Fig. 19). This supported similar findings by Carvalho (1996). The ethanol series alone produced acceptable results with minimal surface cracking. The ethanol/HMDS series, however, produced the best samples for SEM analysis with very minimal surface cracking (Figs.8,9) and good preservation during storage. The ethanol/HMDS technique is also easy to perform. These findings with ethanol/HMDS for dehydration/drying are in agreement with recent studies (Perdigao et al. 1995, Cavalho et al. 1996). Therefore, when dehydrating/drying mineralized dentin samples for scanning electron microscopy, ethanol/HMDS is an effective method.

Since anatomy-dependent regional differences regarding tubule density and size occur (Schellenberg et al. 1992, Dourda et al. 1994) it was important to obtain

tooth and site specific data in this study. Anatomy-dependent regional differences regarding tubule density and size were not recognized by earlier investigators who generally sampled from unspecified teeth and non-specific sites (Tronstad 1973, Garberoglio and Brännström 1976). This led to a wide range in reported tubule density. To determine the number of teeth required for this study, periodic power analysis of the gathered data was performed. The results revealed that additional samples beyond 10 teeth (90 micrographs) would not have a statistically significant effect on the results. To be thorough, a total of twenty teeth were selected for the study. The specified teeth were selected from adolescent patients who required extractions for orthodontic reasons. The specified site was pulpal wall at the apical one-third of the teeth. The coefficient of variation for each level was low. Teeth from a mixed age population were not used to avoid potential variables related to age changes and their effects on dentinal tubule morphology.

Schellenberg et al. (1992) found significantly ($p \leq 0.01$) greater tubule density at the buccal/ lingual wall than at the mesial/distal wall in coronal dentin. Attempts were made to section samples along the mesial/distal plane of intact teeth to view the buccal/ lingual walls and compare these values to the mesial/distal walls which were from sections made along the buccal/lingual plane of the intact teeth. It was not possible to section these samples in the apical region, however. The anatomy and concavity of the pulpal wall in this region did not allow for adequate sectioning. Those samples that were able to be sectioned had a very deep, narrow concave surface that

did not permit proper SEM evaluation. The less concave mesial/distal pulpal wall was a more suitable surface for SEM evaluation.

Evaluation of the dentinal surface with scanning electron microscopy is challenging due to the anatomical location and orientation of the dentinal tubules and the surface morphology of the specimen. It results in a distorted measurement of the number of tubules, tubule diameter and the percentage area these tubules occupy in a given field. Many studies in the literature investigating dentinal tubule dimensions fail to address the measurement errors caused by tubule orientation (Tronstad 1973, Carrigan 1984). Arends (1994) and Garberoglio and Brännström (1976) were the only authors to address this dilemma. Tidmarsh and Arrowsmith (1989) stated their micrographs were "tilt compensated" but did not elaborate on this. Therefore, a method was developed in this study for data collection and analysis to minimize distortion and measurement error caused by orientation problems.

The concave shape of the pulpal wall creates imaging problems when the SEM beam scans the surface. A given sample site of dentinal tubules will have a certain number of tubules oriented directly parallel to the electron beam (the diameter of the tubules is measured perpendicular to the direction of the beam) and others viewed at an angle distorting the actual size of the tubule diameter (Figs.1,2,3,4). The tubules which are parallel allow for a true measurement of diameter size (and thus true percentage area calculation). The tubules viewed at an angle will yield a false value when the diameter is calculated. This value would most likely be smaller than

the actual size value. This would also distort the percentage area calculation. The numerical density measurement would also be affected. Tubules oriented at a severe angle to the beam may appear severely distorted and thus be regarded as artifact.

The standardized random method of site selection for viewing dentinal tubules was reproducible and gave a sufficient representation of the pulpal wall at the given level. This represented a standard random selection of sites within an area of 0.01 mm^2 along the pulpal wall surface. Attempts at using a larger area were not successful. Obvious image distortion of the tubules occurred as the transition region between the opposing walls (ie. buccal to distal wall) was entered and the concavity of the pulpal wall became more pronounced.

In an attempt to improve measurement technique of dentinal tubules, Arends et al. (1995) used video image acquisition from a macrolens and a video camera to magnify their images (10-20X). Bochacki et al. (1992) however, determined that video based image analysis results in image distortion around the periphery of the video screen. This distortion, although significant ($p \leq 0.01$) was fairly small (1.07%).

The data collection and analysis method was performed with digitized micrograph images. Flat-bed scanned image acquisition systems provide an accurate digital image (Bochacki et al 1992). Digital imaging offers several advantages over conventional film prints when measuring and counting dentinal tubules. The images, analyzed with Adobe Photoshop, could be visually enhanced by adjusting brightness/contrast levels, sharpness and improving grey scale shades. Most

importantly, magnification settings could be adjusted. This allows for very accurate selection of tubule diameter and number, because the exact peripheral border of the tubule could be accurately visualized. There was very little difficulty defining the exact diameter of the tubule and imaging artifact was ruled out. For example, when selecting tubules for *Mask 2,* the image could be divided into four quadrants on the computer screen. Each quadrant could be magnified. The exact circumference of a given tubule could be measured by magnifying the tubule and viewing the pixel shades. This method was very time consuming but provided a very accurate method for tubule measurement.

Several studies examining tubule density and diameter have reported large variation among samples. This is most likely the result of non-site and non-tooth specificity among those tooth samples (Schellenberg et al. 1992). Also, the method by which samples are prepared for SEM evaluation is also important (Arends et al. 1995, Carvalho et al. 1996). These factors were addressed in this study and resulted in consistent data ($p \leq 0.0001$) regarding dentinal tubule measurements. Mineralized dentin was used in all tooth specimens. These teeth were selected from a population of similarly aged individuals. Specific tooth sites were examined in the apical region using a standard random selection method. This method utilized the application of digital imaging for data collection which yielded consistent results with very little artifact..

The creation and application of *Mask 1* resulted in an accurate calculation of numerical tubule density. The measurement of tubule diameter was accomplished by increasing the magnification of the digital image and recording the exact periphery of the tubule as previously mentioned. *Mask 2* was then created resulting in an average diameter size for those tubules in the image field which were properly oriented for viewing. This value was then applied to the total number of tubules in the image field to calculate the average tubule diameter for that level (ie. apical 1 mm). This method compensated for both severely distorted tubules and image artifact. The low p values (0.0001) are a direct result. These consistent average diameter values were applied to percentage area calculations and low p values (0.0001) also resulted. Therefore, distortion and measurement error due to specimen tilt and orientation of tubules are minimized with the selection and data collection methods described in this study.

The accuracy of these methods was tested in a third pilot study. Three groups were created (A, B, and C) to compare tubule measurements of different orientations of the SEM stage relative to the SEM beam. This was to simulate the actual anatomical location and orientation of the tubules relative to the beam and would be accomplished by tilting the SEM stage where the specimen is scanned by the beam. All groups (A, B and C) examined the same tooth segment location but each were at different stage tilt settings. In group A, the stage tilt setting was set at 0° which oriented the specimen surface perpendicular to the electron beam of the SEM. The tilt for group B was set at -4° (-4° from the group A setting). These specimens were tilted 4° counter-clockwise relative to the electron beam. In group C, the stage tilt was +4°

tilt (+4° from the group A setting) and the specimens were tilted 4° clockwise relative to the electron beam. Thus, relative to the electron beam, a given sample was viewed from three different angles. Specific dentinal tubules would be viewed straight on at one angle, and would be distorted at the other angles. The accuracy of the selection method previously described was then tested using one-way analysis of variance and Levene test for homogeneity of variances. Data were compared among the three tilt settings for each sample and not among different samples. There was no significant difference ($p < 0.001$) when comparing tubule dimensions at the three different stage tilt settings for a given sample. Standard deviations were minimal in the majority of the 21 sites studied at each level.

In exposed dentin, the outward fluid flow through the dentinal tubules is an important defense mechanism against the inward diffusion of noxious substances (Pashley 1996). This outward fluid flow is not present in endodontically treated teeth. because positive pulpal pressure is absent. The permeability of dentin is a function of tubule density and their diameters (Pashley and Pashley 1991). The rate of diffusion of materials through dentin is directly proportional to the percentage area occupied by tubules (Pashley 1988). Pashley (1996) has shown that dentinal tubules are major channels for solute diffusion and movement of fluid across dentin between periphery and pulp. Leakage of microorganisms and their toxins through exposed dentinal tubules into the surrounding tissues is a concern associated with endodontic surgery. Inefficient retrograde sealing of the root canal following root-end resection has been suggested as being a major factor in surgical endodontic failure (Rud et al. 1972).

The results of this study indicate a significant difference in tubule number and size especially between the apical 1 mm and 6 mm levels. This is an important consideration in regard to endodontic surgical techniques, especially root-end preparation. This poses a question in regard to exposed dentinal tubules after root resection and the creation of a beveled root surface prior to root-end filling placement. An increase in both the amount of root resected and the degree of bevel angulation created significantly influence apical leakage (Gilheany et al. 1994).

The application of recent surgical techniques and armamentarium such as the surgical operating microscope and ultrasonic instrumentation systems allow for a more conservative root-end preparation. Further studies are required to investigate the role of apical microleakage associated with exposed dentinal tubules after root-end preparation and its role in periapical surgery prognosis. This information may have application to endodontic surgery techniques in the future.

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TABLE 1

PILOT STUDY III DATA

Level 1 = 1mm from apex
Level 2 = 3mm from apex
Level 3 = 6mm from apex

LEVEL	TOOTH	ANGLE	DIAMETER	AREA	TUB/mm2
1	1	0	1.07	2.95	17535
1	1	4	1.10	2.90	16826
1	1	-4	1.22	3.21	16724
1	2	0	1.21	3.34	16319
1	2	4	1.24	3.11	15914
1	2	-4	1.21	3.04	15914
1	3	0	1.04	2.41	14697
1	3	4	1.07	2.56	15305
1	3	-4	1.08	2.62	15500
1	4	0	1.12	3.19	18244
1	4	4	1.13	3.37	18954
1	4	-4	1.16	3.28	18042
1	13	0	1.20	3.35	17231
1	13	4	1.20	3.20	17028
1	13	-4	1.22	3.21	16724
1	14	0	1.22	3.01	16014
1	14	4	1.24	3.11	15914
1	14	-4	1.23	3.09	16015
1	15	0	1.08	2.62	15508
1	15	4	1.10	2.73	15812
1	15	-4	1.13	2.84	16015
2	5	0	1.23	7.07	36489
2	5	4	1.25	7.36	37605
2	5	-4	1.27	7.29	36692
2	6	0	1.27	7.34	36794
2	6	4	1.27	7.19	36084
2	6	-4	1.27	7.42	37097
2	7	0	1.22	6.75	35273

Level 1 = 1mm from apex					
Level 2 = 3mm from apex					
Level 3 = 6mm from apex					
LEVEL	**TOOTH**	**ANGLE**	**DIAMETER**	**AREA**	**TUB/mm2**
2	7	4	1.24	7.01	36084
2	7	-4	1.22	6.78	35374
2	8	0	1.30	7.88	38517
2	8	4	1.29	7.63	36794
2	8	-4	1.28	7.70	38314
2	16	0	1.23	7.07	36489
2	16	4	1.25	7.32	37300
2	16	-4	1.21	6.76	35577
2	17	0	1.29	7.88	38517
2	17	4	1.32	8.04	38820
2	17	-4	1.34	7.89	37503
2	18	0	1.22	6.78	35374
2	18	4	1.25	6.82	34766
2	18	-4	1.21	6.83	35982
3	9	0	1.69	12.64	47639
3	9	4	1.66	12.92	49565
3	9	-4	1.64	12.58	48855
3	10	0	1.55	12.16	49970
3	10	4	1.57	12.46	50477
3	10	-4	1.53	12.24	50882
3	11	0	1.63	12.18	47639
3	11	4	1.60	12.26	48754
3	11	-4	1.62	12.58	49463
3	12	0	1.66	13.20	50781
3	12	4	1.60	12.92	51389
3	12	-4	1.63	12.90	50477
3	19	0	1.67	12.62	48146
3	19	4	1.69	12.64	47639
3	19	-4	1.65	12.6	48653
3	20	0	1.56	12.31	50274

Level 1 = 1mm from apex					
Level 2 = 3mm from apex					
Level 3 = 6mm from apex					
LEVEL	**TOOTH**	**ANGLE**	**DIAMETER**	**AREA**	**TUB/mm2**
3	20	4	1.57	12.46	50477
3	20	-4	1.54	12.13	50173
3	21	0	1.63	12.97	50680
3	21	4	1.66	13.2	50781
3	21	-4	1.64	13.1	50882

Apical Level	Mean	SD	Range	95%Confid. Interval for the Means	No. of Samples
1 mm	17,067	1845	14,393 - 21,589	16,718 - 17,415	110
3 mm	36,623	3810	28,380 - 58,921	35,945 - 37,300	125
6 mm	48,440	614	38,516 - 69,533	47,352 - 49,527	125

Table 2: Dentinal Tubule Density (Tubules/mm^2). Results of the average numerical tubule density (Tubules/mm^2) at the three apical levels.

Apical Level	Mean	SD	Range	95% Confid. Interval for the Means	No. of Samples
1 mm	2.95	0.69	1.47-4.91	2.82 - 3.08	110
3 mm	7.27	1.25	1.28-10.03	7.05 - 7.50	125
6 mm	11.57	1.75	7.62-14.94	11.26 - 11.88	125

Table 3: Percentage Area of Dentinal Tubules (% area). Results of the average percentage area occupied by dentinal tubules (% area) at the three apical levels.

Apical Level	Mean	SD	Range	95%Confid. Interval for the Means	No. of Samples
1 mm	1.10	0.24	0.80 - 2.01	1.05 - 1.14	110
3 mm	1.28	0.11	0.97- 1.57	1.25 - 1.30	125
6 mm	1.52	0.14	1.26- 1.77	1.49 - 1.54	125

Table 4: Diameter of Dentinal Tubules (μm). Results of the average diameter of dentinal tubules (μm) at the three apical levels.

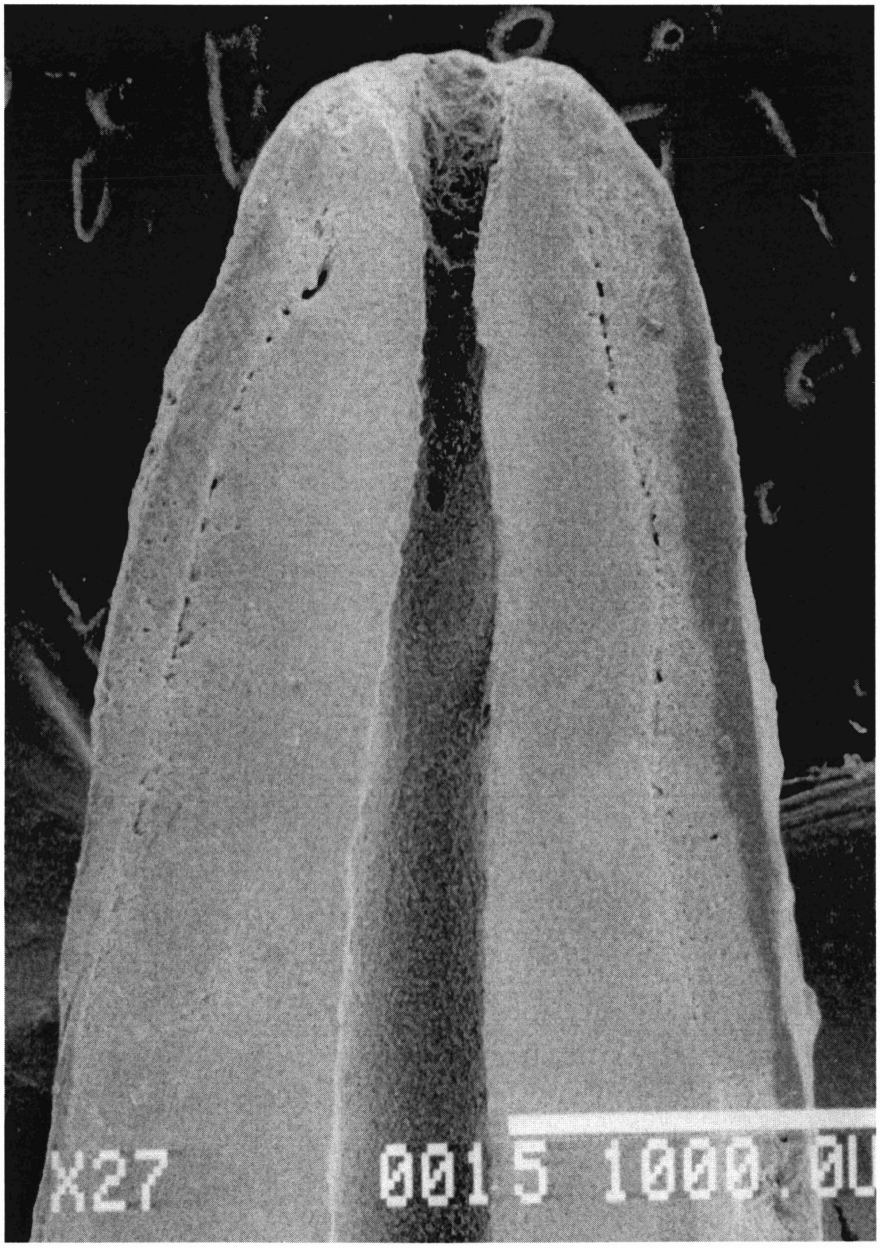


FIG. 1: SEM ANALYSIS OF TOOTH SEGMENTS. Scanning EM view of the distal wall of the pulp chamber at the apical level of a mandibular premolar. Original magnification is 27X.

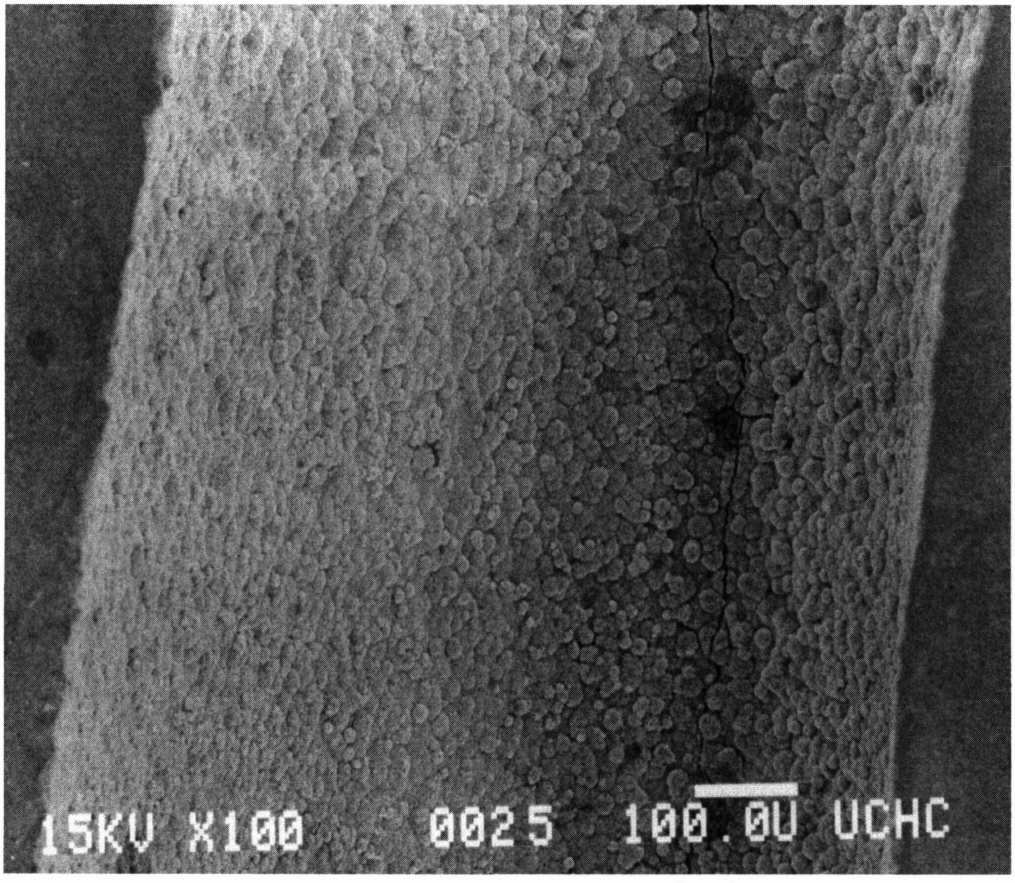


FIG. 2: SEM ANALYSIS OF TOOTH SEGMENTS. Scanning EM view of the pulpal wall at the apical 6mm level of a mandibular premolar. Note the concave shape of the surface. The original magnification is 100X.

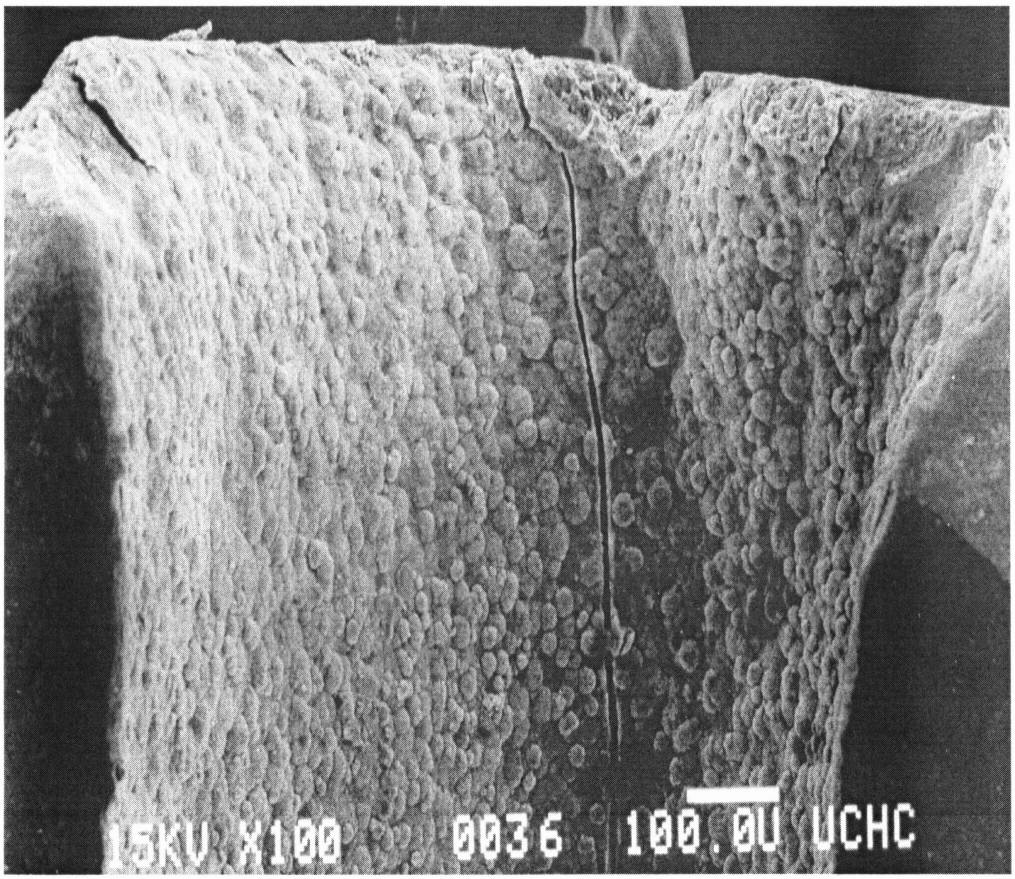


FIG. 3: SEM ANALYSIS OF TOOTH SEGMENTS. Scanning EM view of the pulpal wall at the apical 0.5mm level of a mandibular premolar. Note the concave shape of the surface and difference in tubule orientation of the left side of the micrograph vs. the center. The original magnification is 100X.

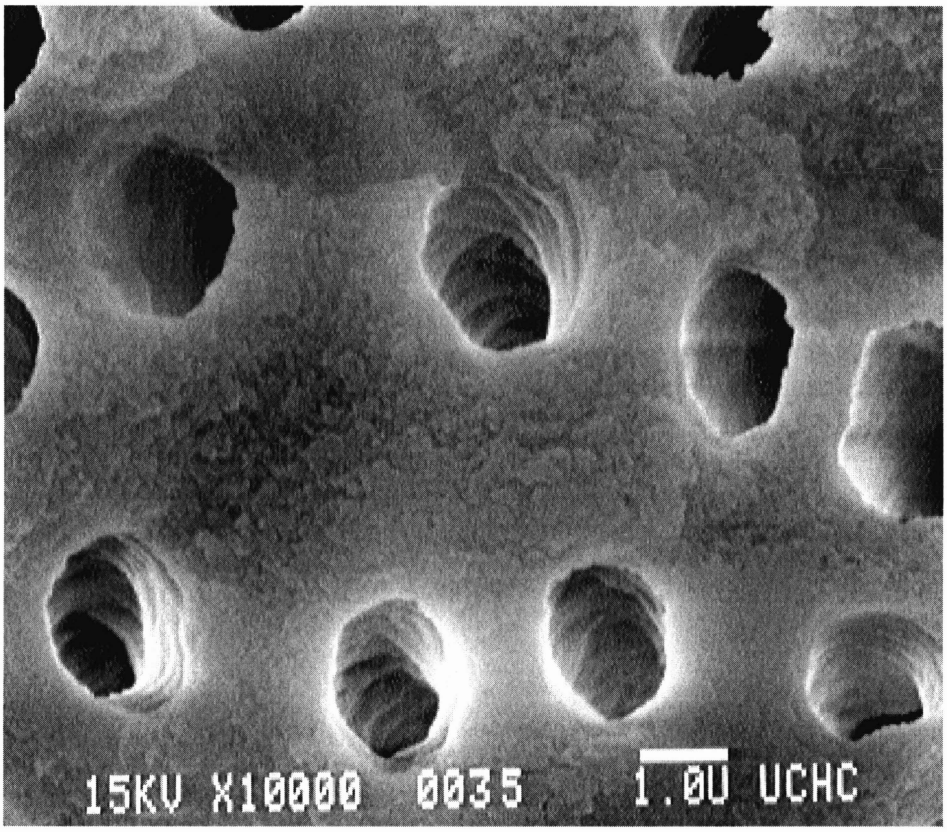


FIG. 4: SEM ANALYSIS OF TOOTH SEGMENTS. High magnification view (10,000X) of dentinal tubules at the apical 6 mm level. Note the differing orientations of the dentinal tubules within the field.

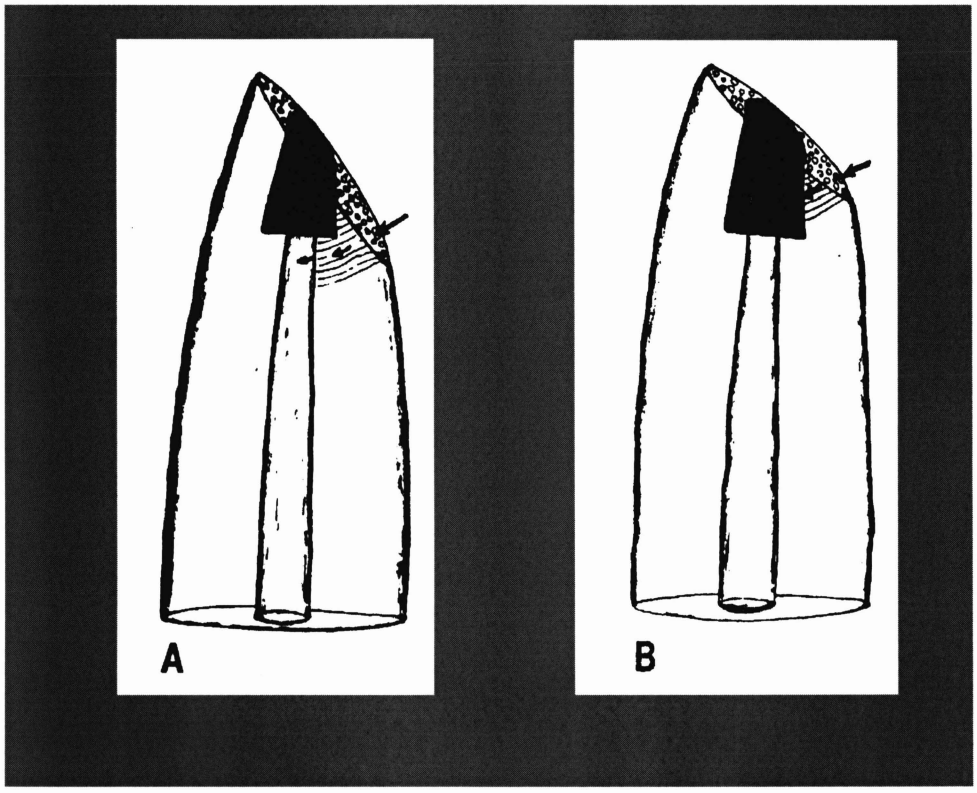


FIG. 5: THE EFFECT OF BEVEL ANGULATION ON EXPOSURE OF DENTINAL TUBULES.
From Vertucci J, Beatty R (1986) Apical leakage associated with retrograde techniques: A dye study. J Endodon 12; 331-336.

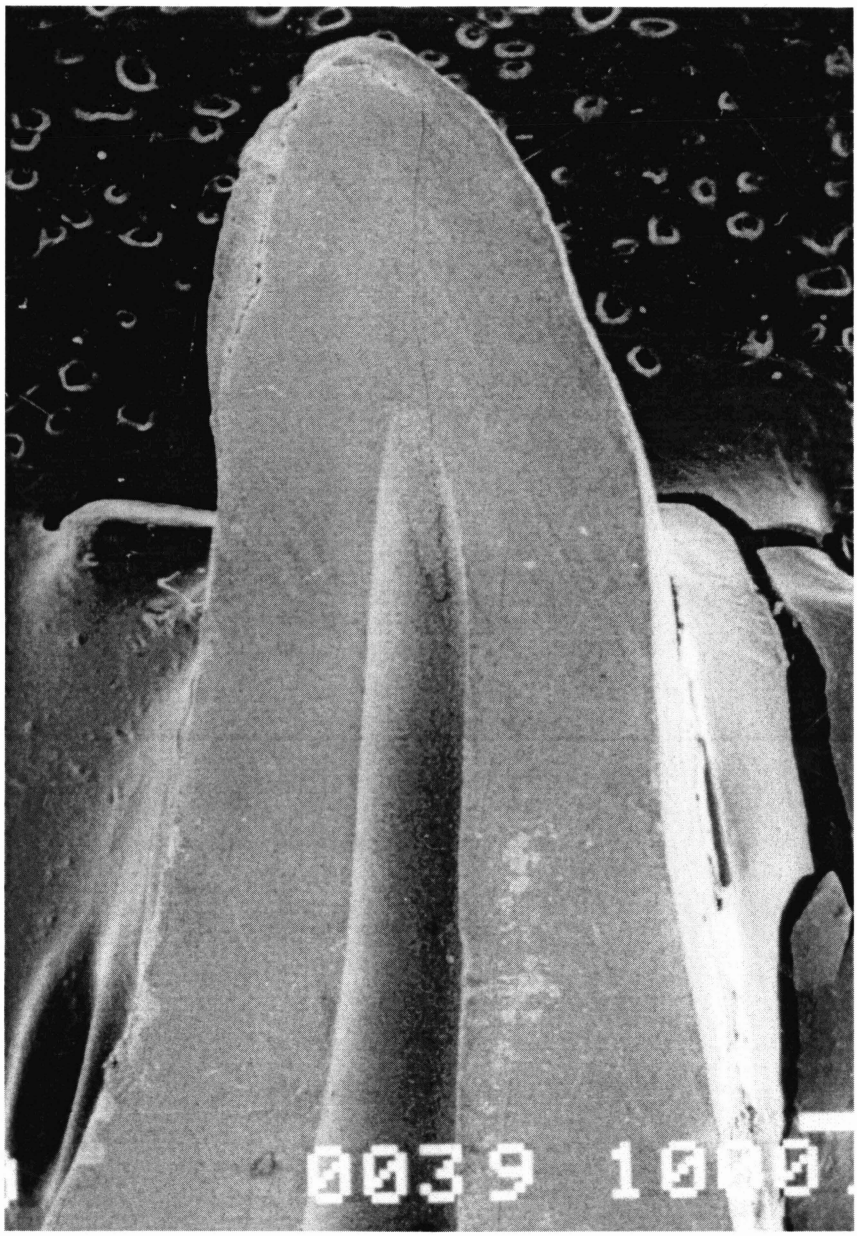


FIG. 6: SECTIONING OF TOOTH SEGMENT WITH A DIAMOND DISC SAW. The straight cutting plane of the blade did not allow for proper sectioning at the apical level of this minimally curved root. The original magnification is 10X.

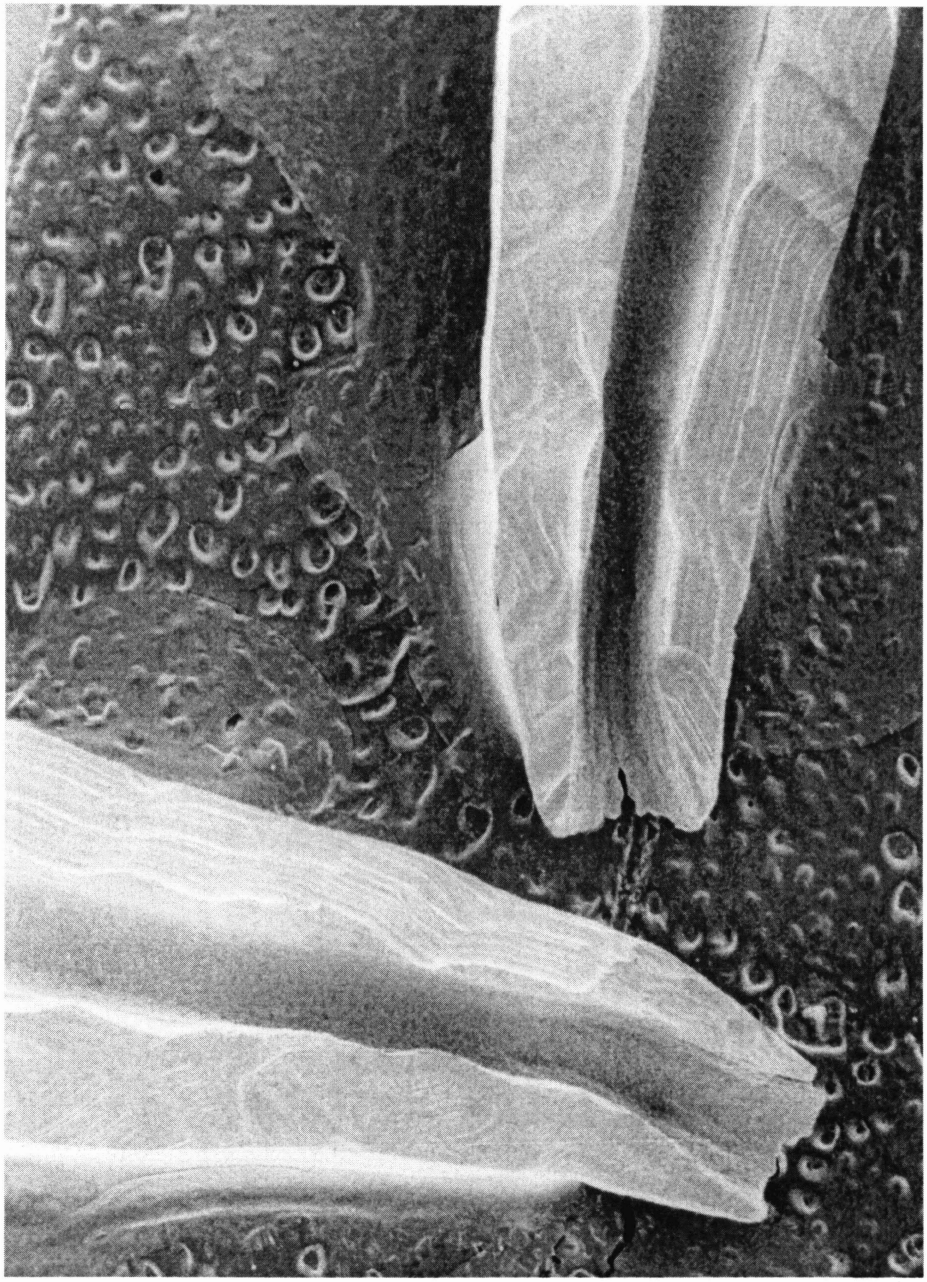


FIG. 7: SECTIONING OF TOOTH SEGMENT. A minimally curved root was properly sectioned along the buccal/lingual plane with the mallet/chisel method. The original magnification is 10X.

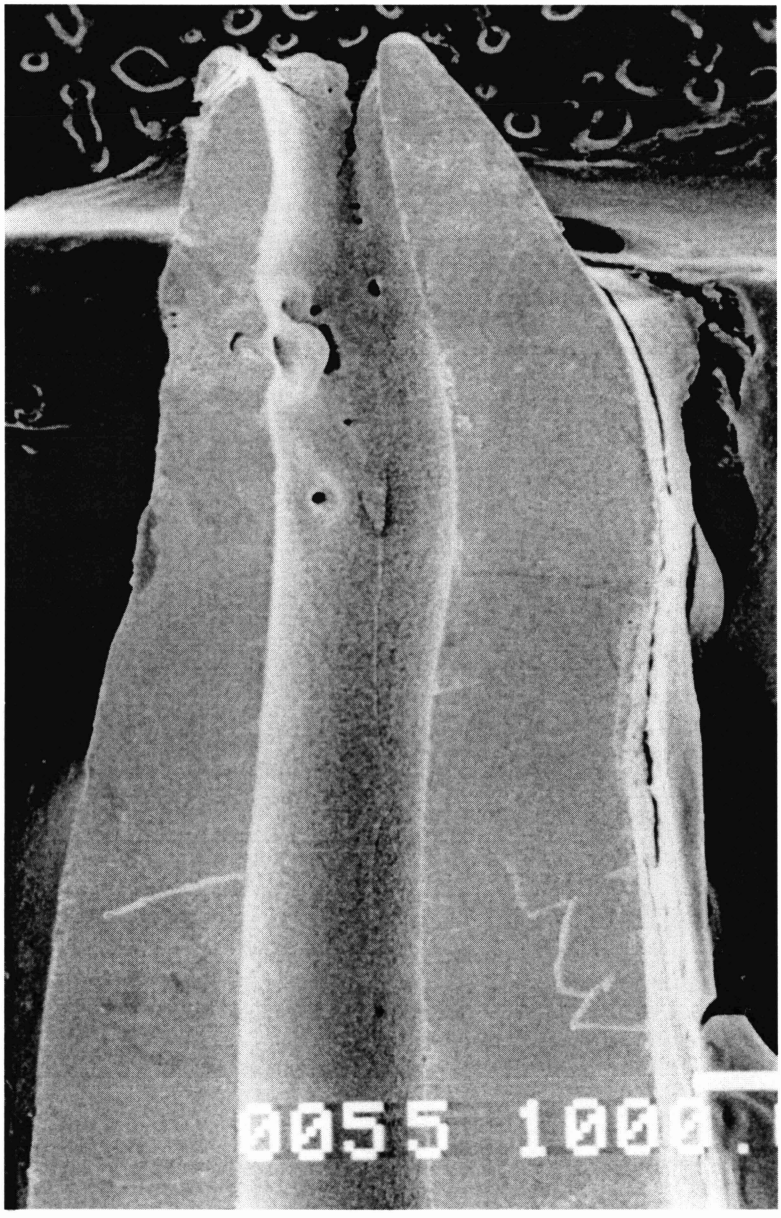


FIG. 8: ETHANOL/ HMDS DRYING METHOD.
A tooth segment dehydrated with ethanol and dried with HMDS. Very minimal surface cracking of the sample surface was noted. The original magnification is 10X.

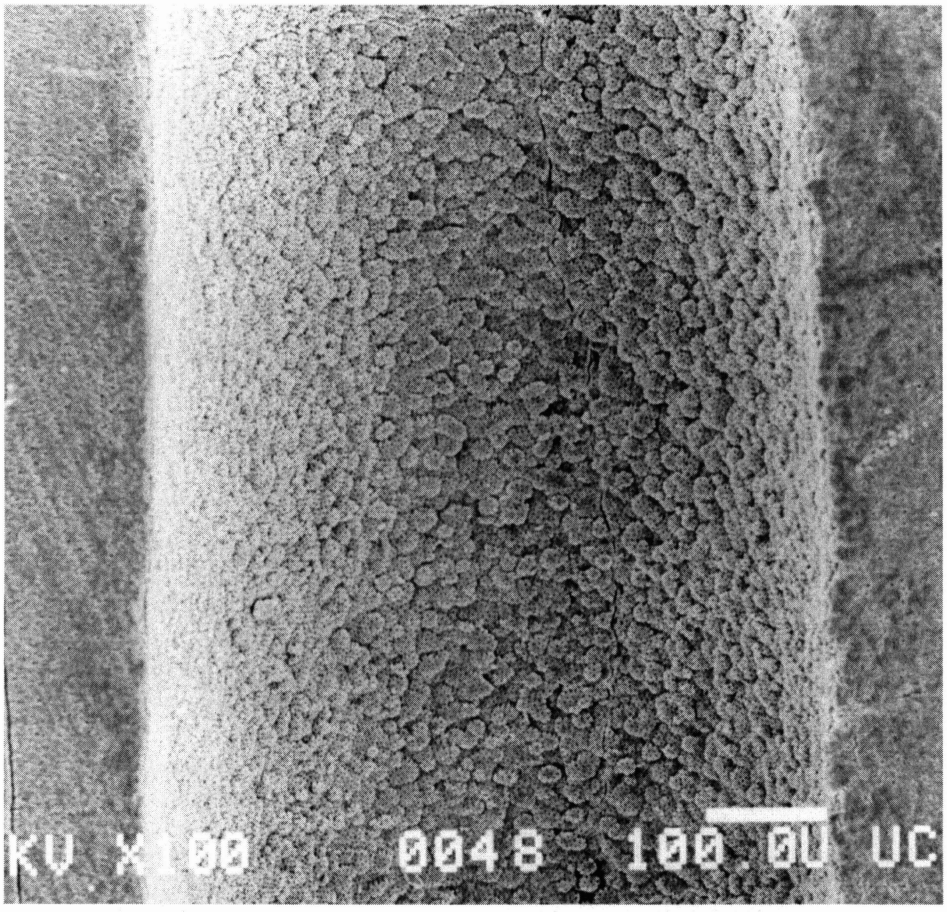


FIG. 9: ETHANAOL/HMDS DRYING METHOD. A tooth segment dehydrated with ethanol and dried with HMDS. Very minimal surface cracking of the sample surface was noted. The original magnification is 100X.

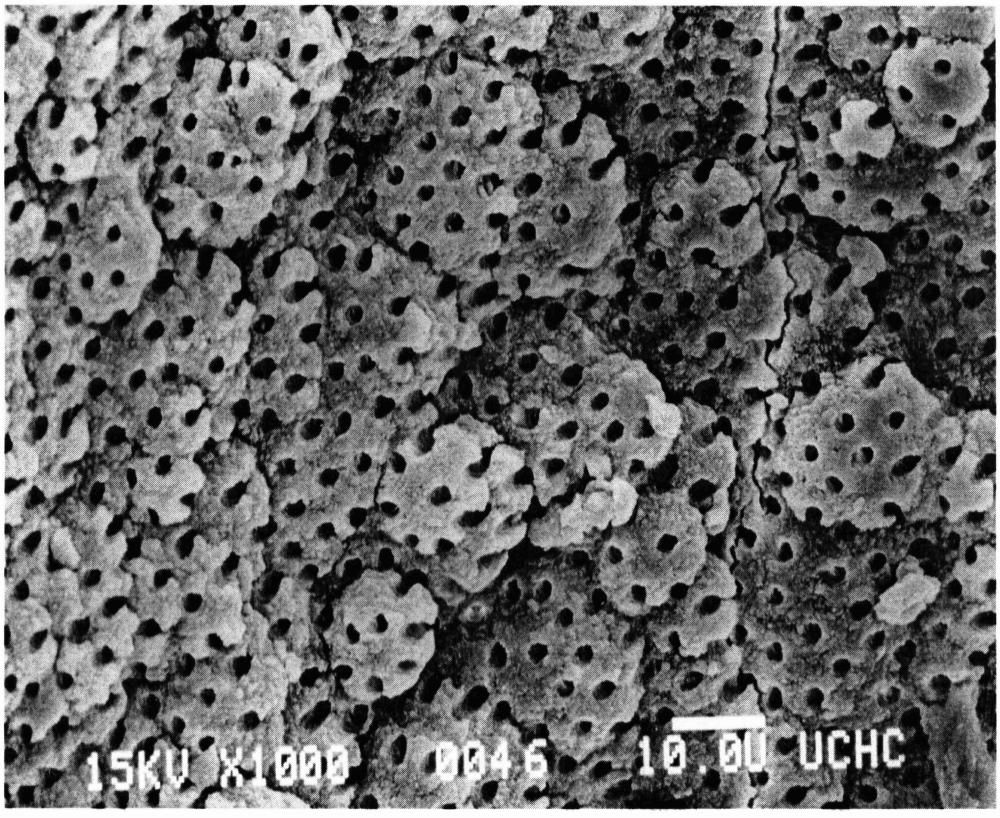


FIG. 10: MAGNIFICATION SETTING. Standardized random method of tubule selection using a magnification setting of 1000X was applied to an inspection site at a known apical level (ie. 6mm).

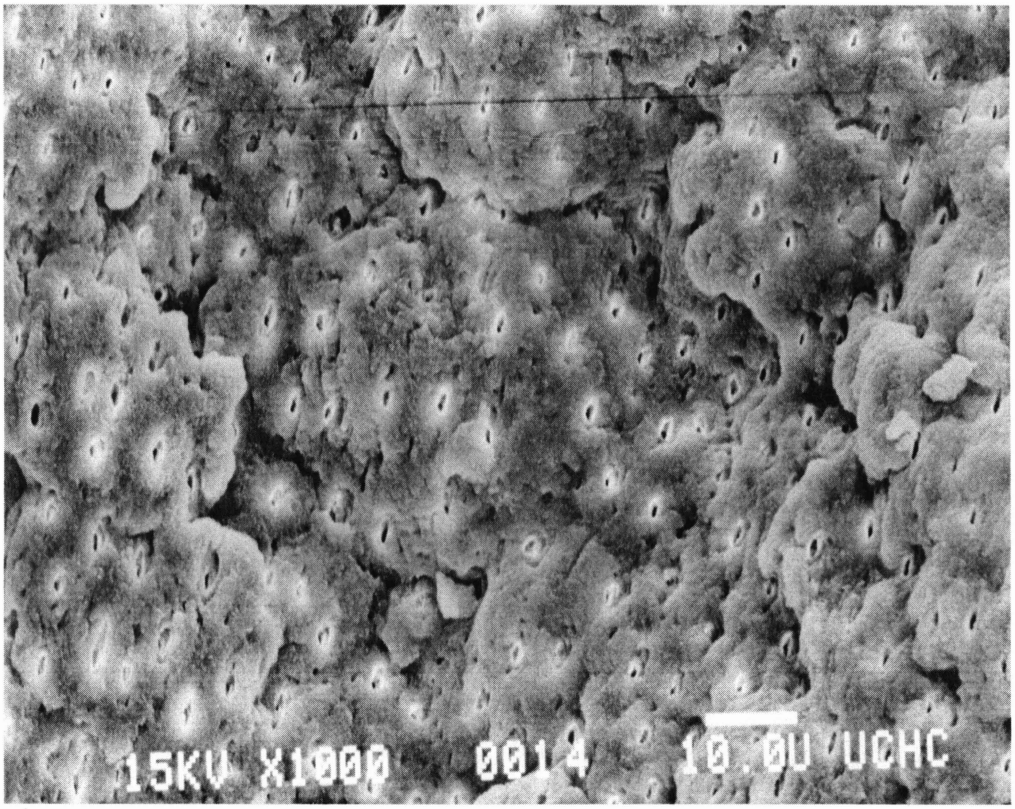


FIG. 11: APICAL 1 MM LEVEL. Tubule Selection at the apical 1 mm level of a tooth segment. The original magnification is 1000X.

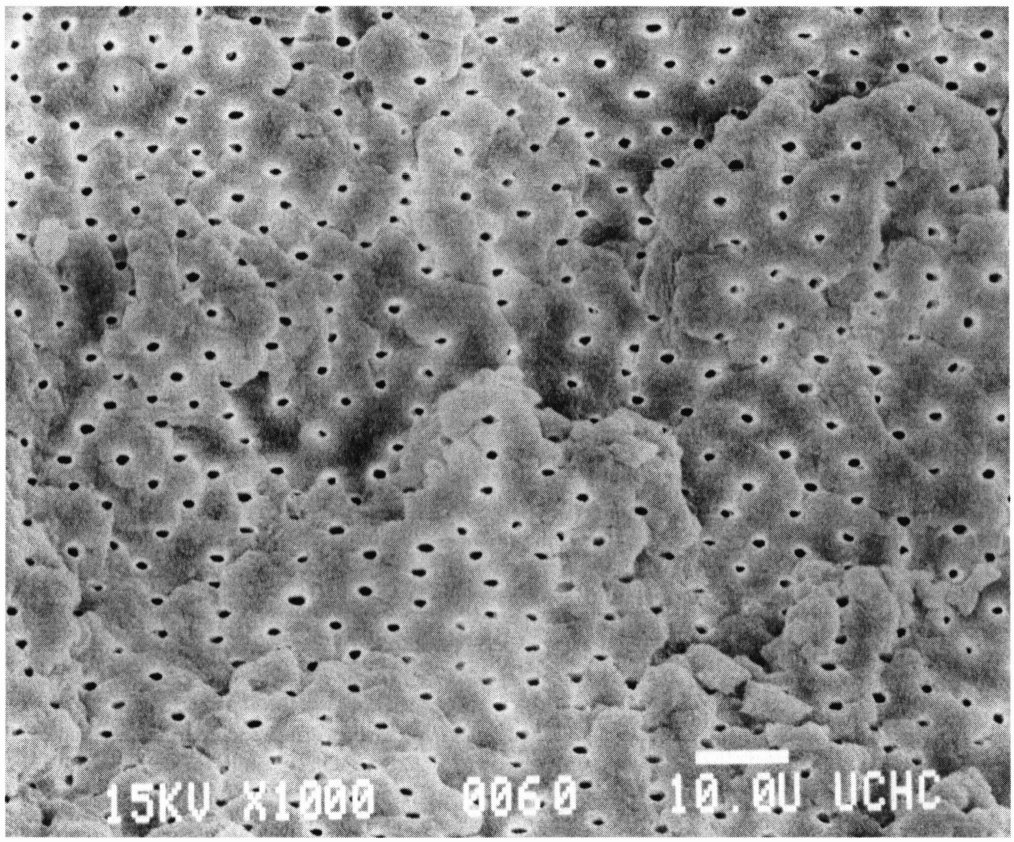


FIG. 12: APICAL 3 MM LEVEL. Tubule Selection at the apical 3 mm level of a tooth segment. The original magnification is 1000X.

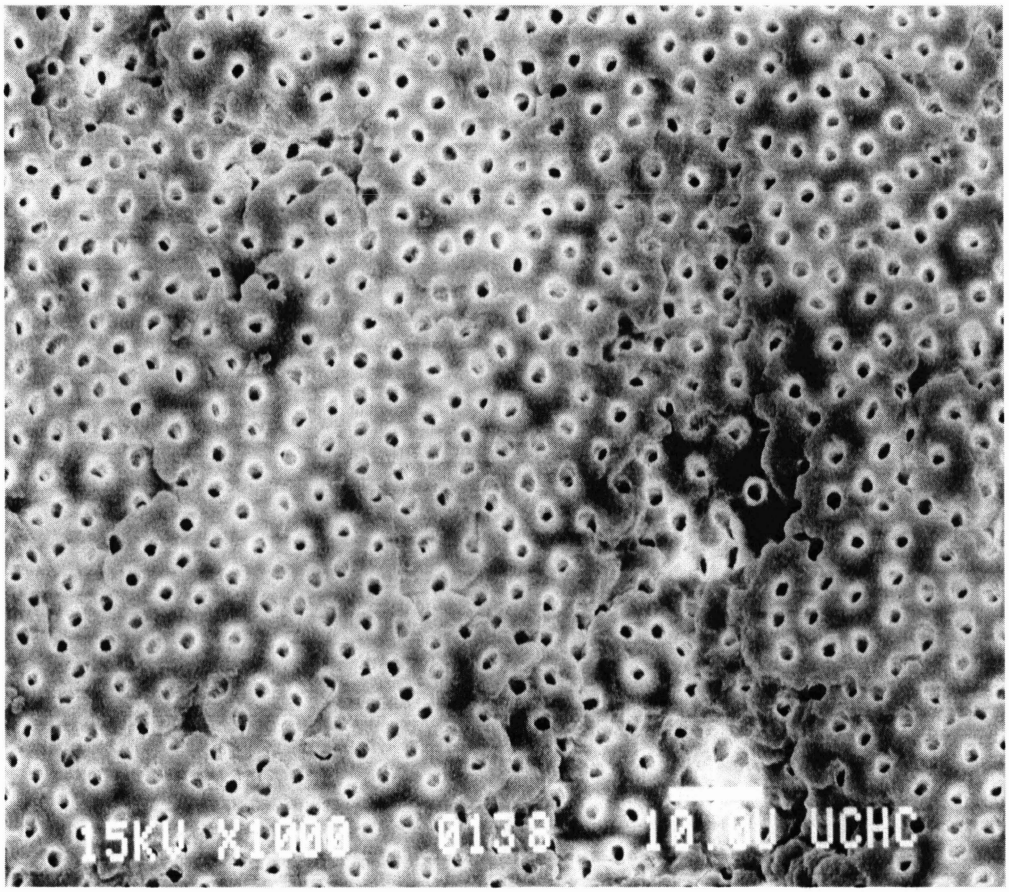


FIG. 13: APICAL 6 MM LEVEL. Tubule Selection at the apical 6 mm level of a tooth segment.

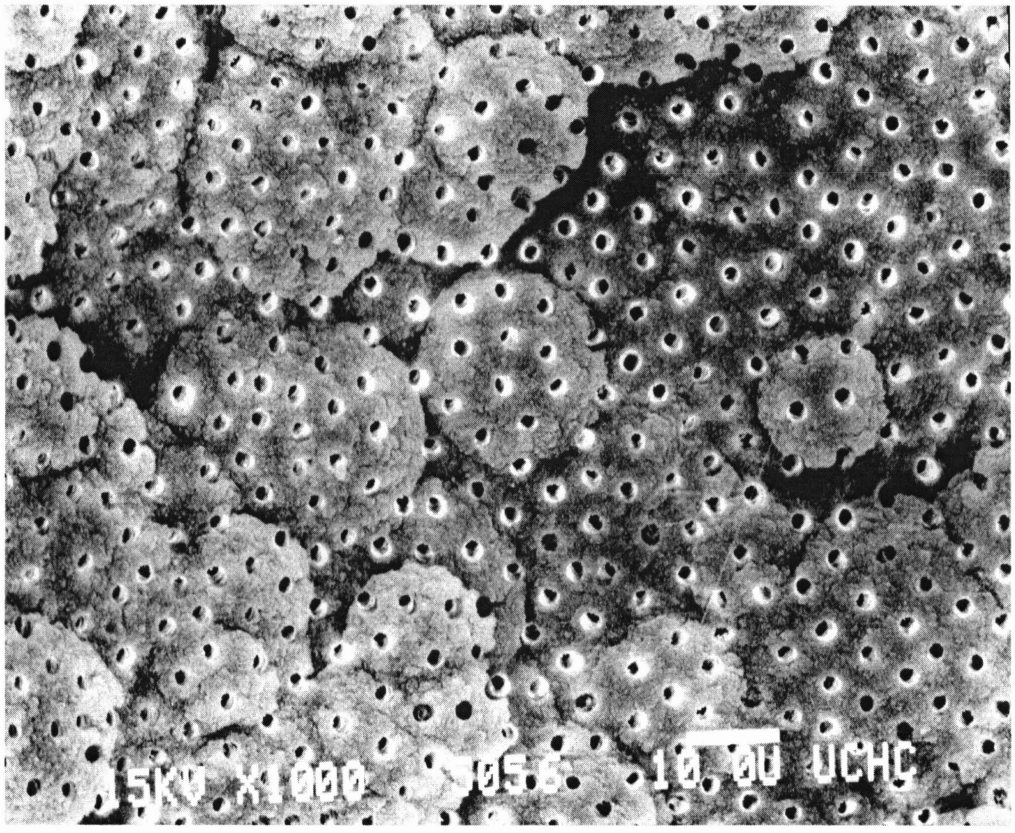


FIG. 14: DENTINAL TUBULE SELECTION AND MEASUREMENT METHOD. The dentinal tubules are dark shades (black) at 1000X magnification. Note areas of artifact adjacent to the dentinal tubules.

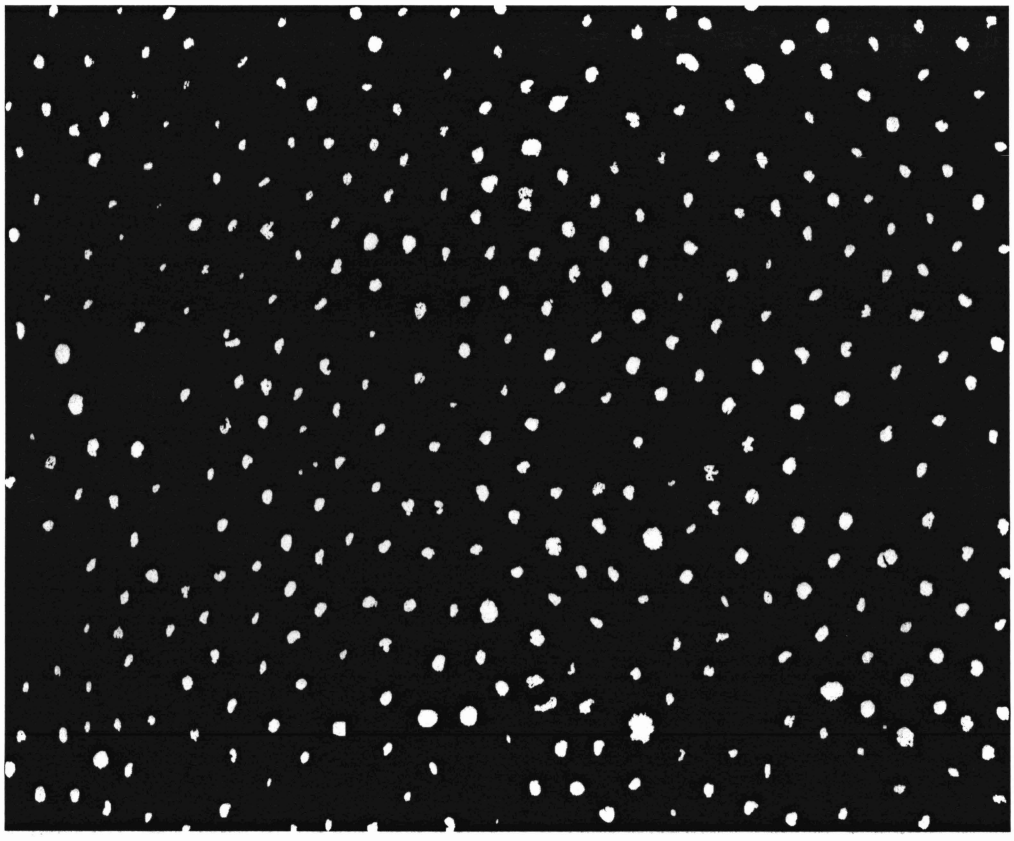


Fig. 15: DENTINAL TUBULE SELECTION AND MEASUREMENT METHOD - MASK 1. The dentinal tubules have been selected out from Figure 14(SEM micrograph) and inverted using Adobe Photoshop to create white "tubules" against a black background for easier site inspection. The original magnification is 1000X.

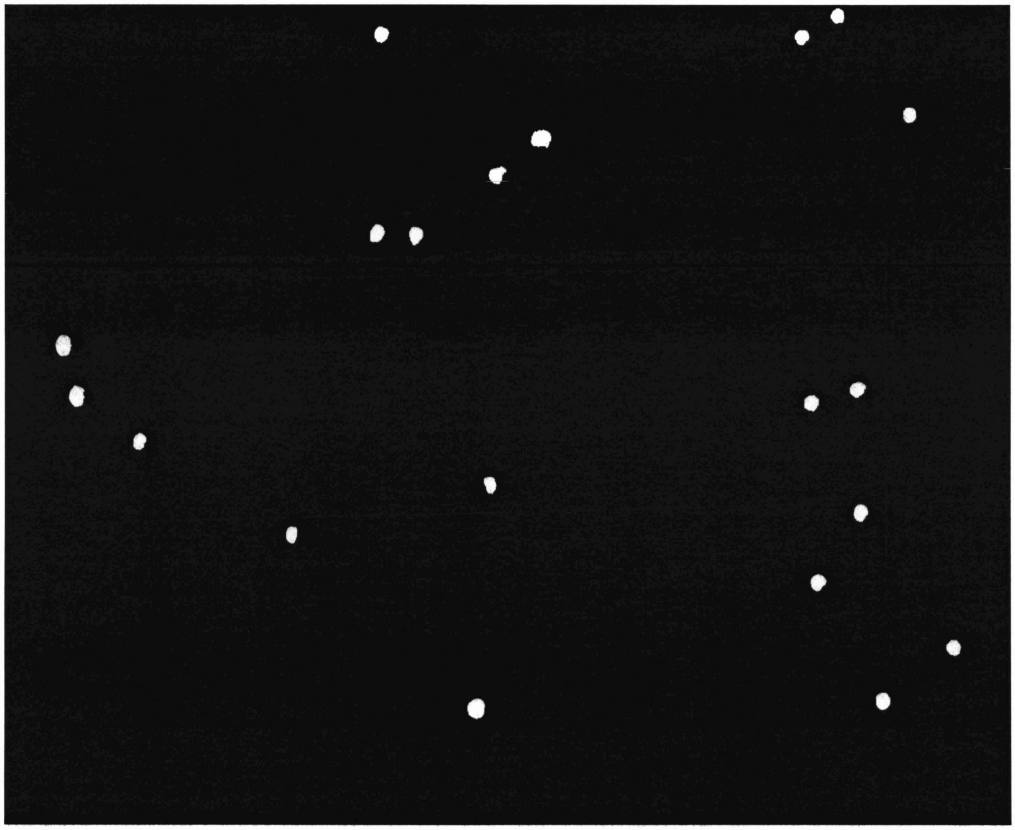


Fig. 16: DENTINAL TUBULE SELECTION AND MEASUREMENT METHOD- MASK 2. Dentinal tubules viewed straight on with respect to the electron beam of the SEM have been selected. The original magnification is 1000X.

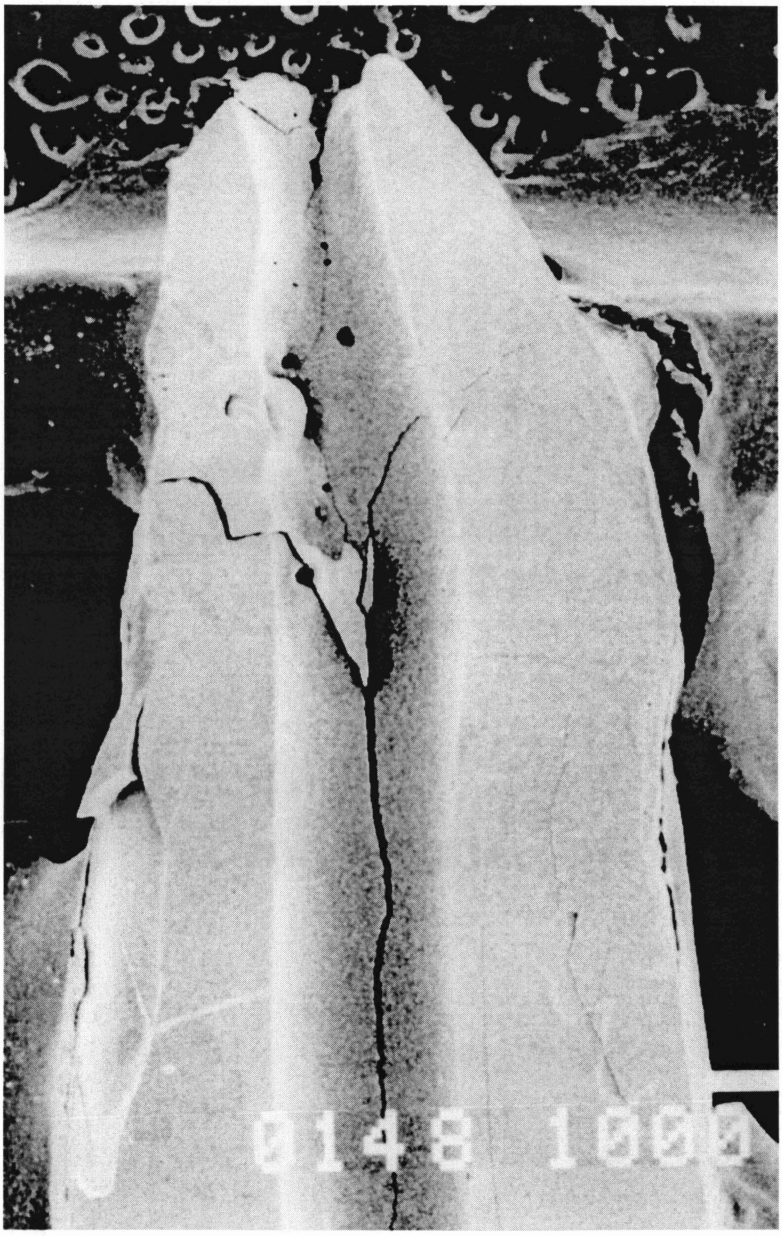


FIG. 17: ETHANOL / CRITICAL POINT DRYING METHOD. A tooth segment dehydrated with ethanol and dried with critical point drying. Significant surface cracking of the sample was noted. The original magnification is 10X.

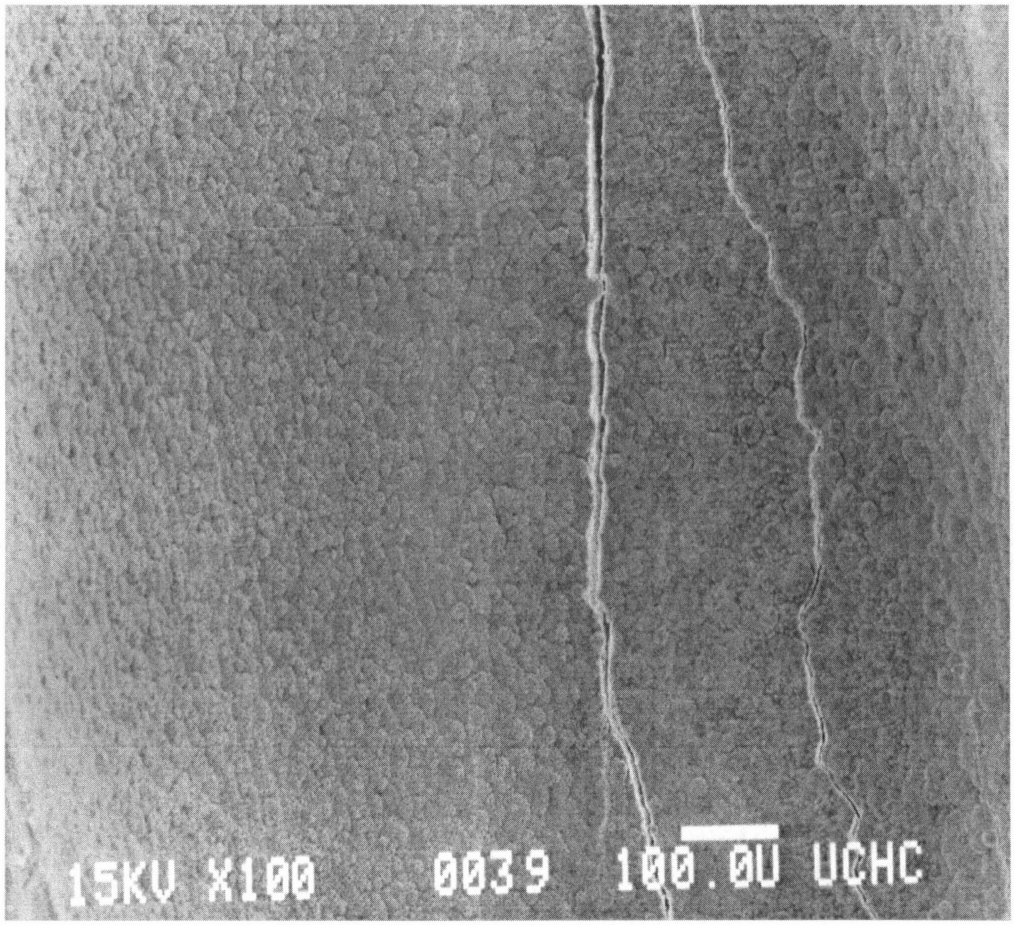


FIG. 18: ETHANOL / CRITICAL POINT DRYING METHOD. A tooth segment dehydrated with ethanol and dried with critical point dryer. Significant surface cracking of the sample is noted. The original magnification is 100X.

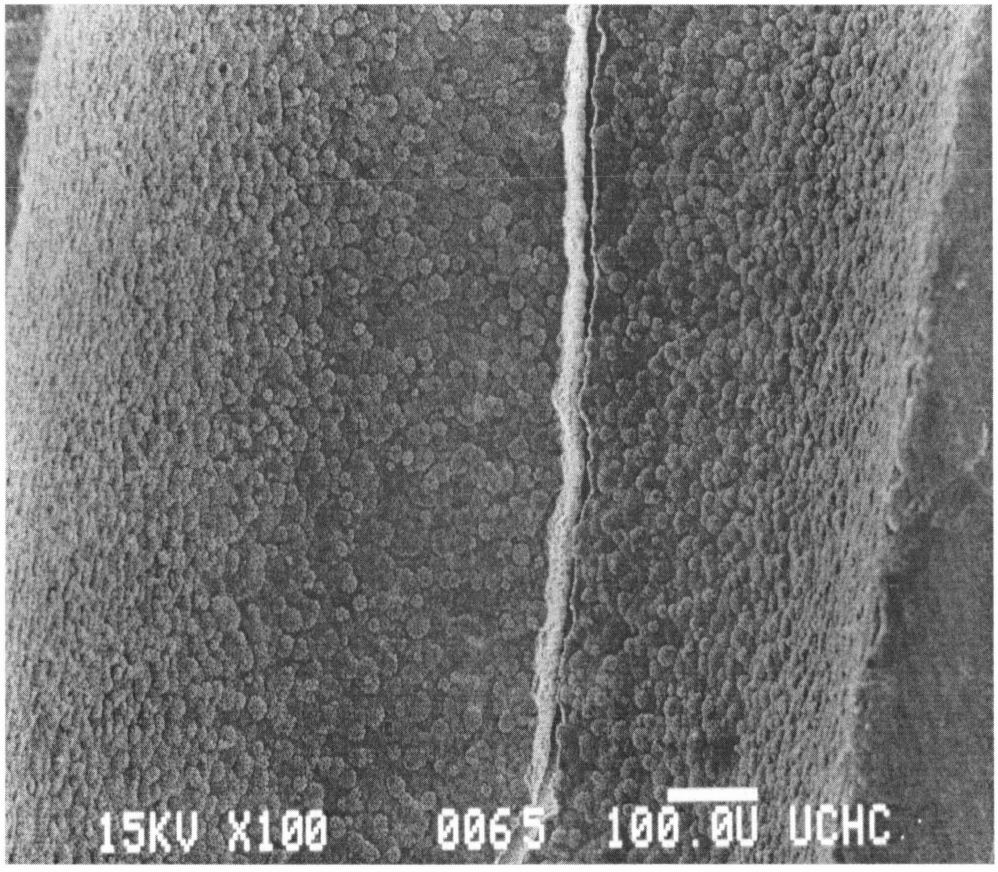
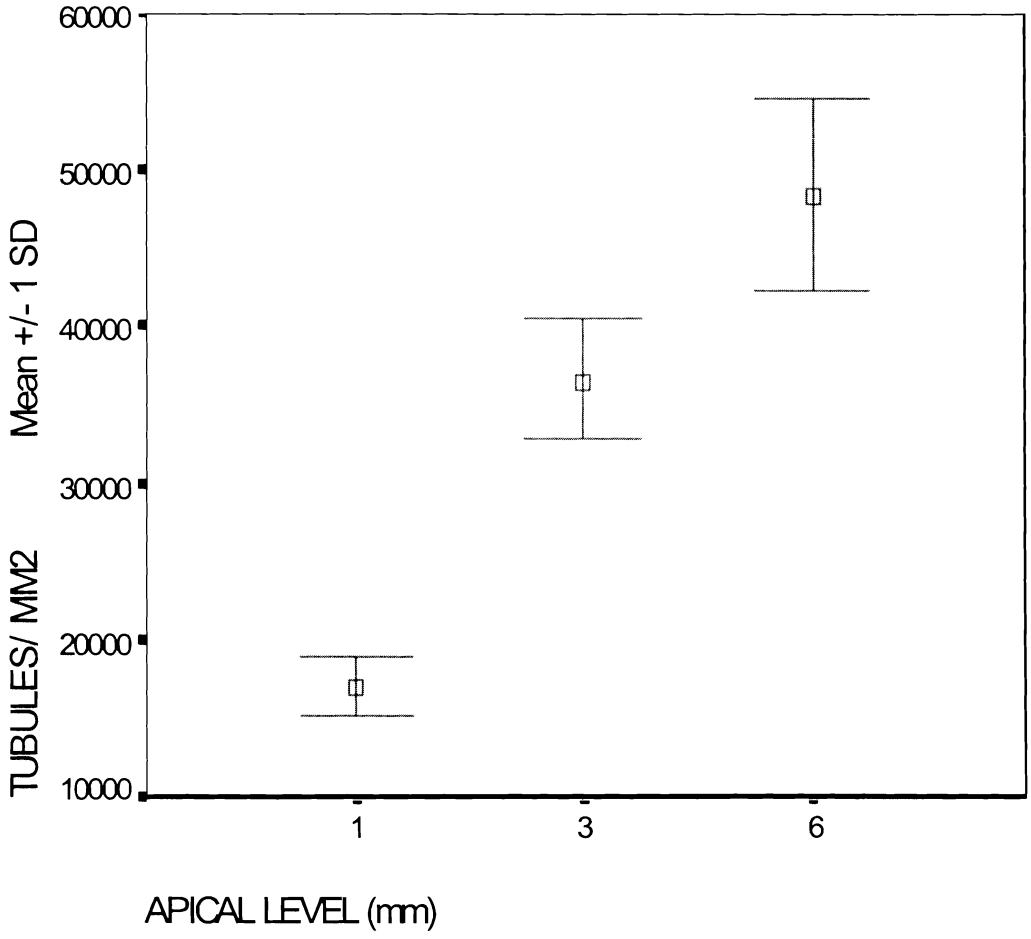


FIG. 19: ETHANOL/ CRITICAL POINT DRYING METHOD. A tooth segment dehydrated with ethanol and dried with a critical point dryer. Significant surface cracking of the sample is noted and this increased with time during storage. The original magnification is 100X.

TUBULE DENSITY (# / mm^2)

Fig. 20



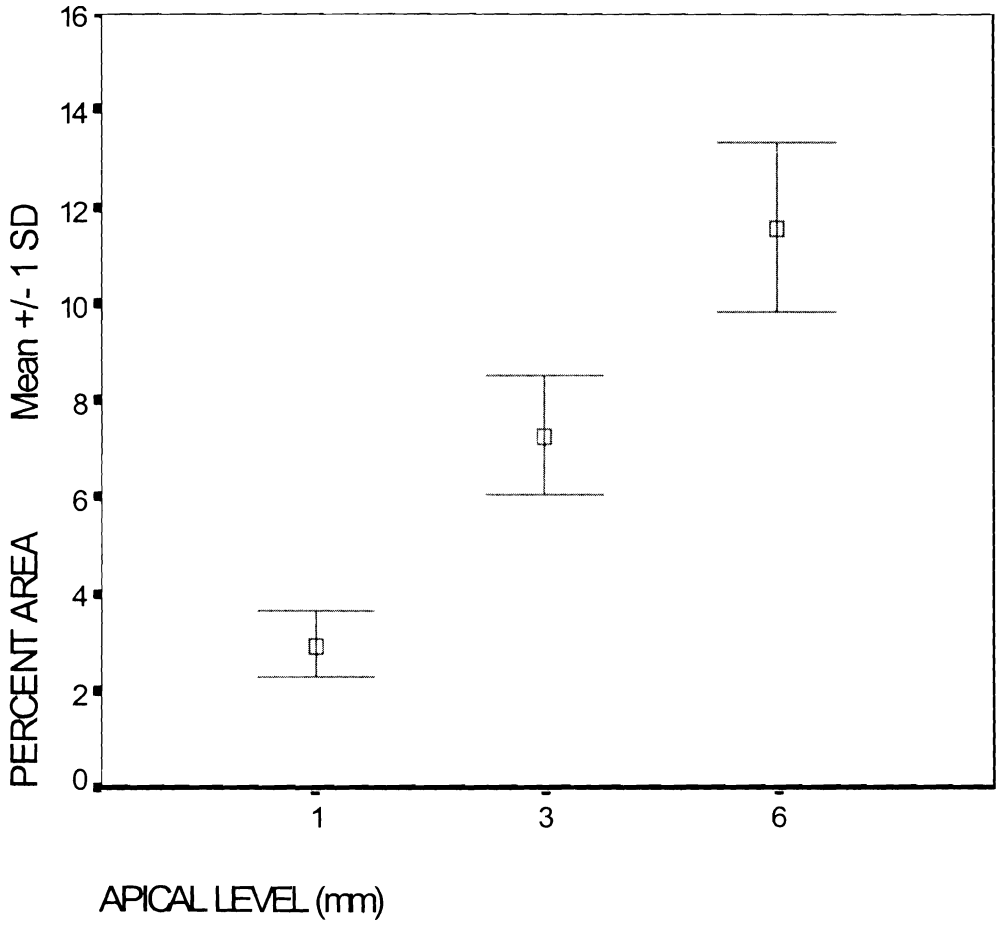
Apical 1 mm level N = 110

Apical 3 mm level N= 125

Apical 6 mm level N = 125

PERCENTAGE AREA OF TUBULES (% AREA)

Fig. 21



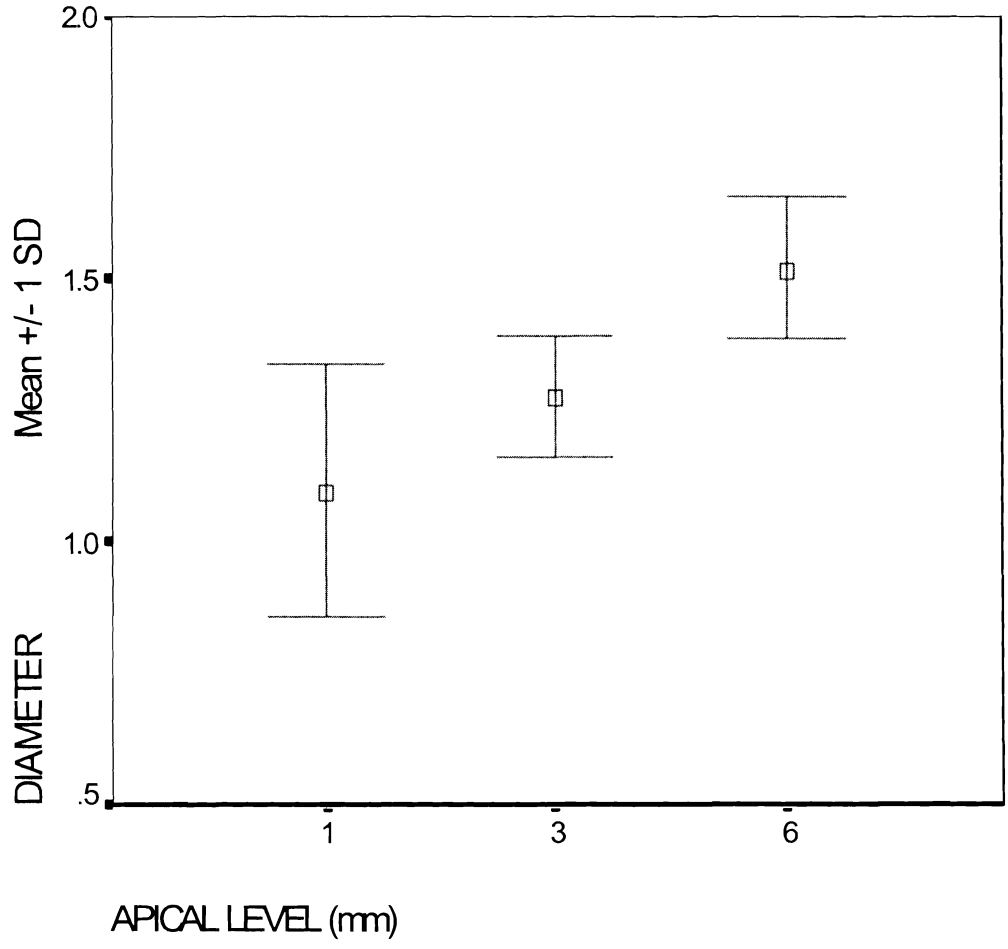
Apical 1 mm level N = 110

Apical 3 mm level N = 125

Apical 6 mm level N = 125

DIAMETER OF TUBULES (μm)

Fig. 22



Apical 1 mm level N = 110

Apical 3 mm level N = 125

Apical 6 mm level N = 125

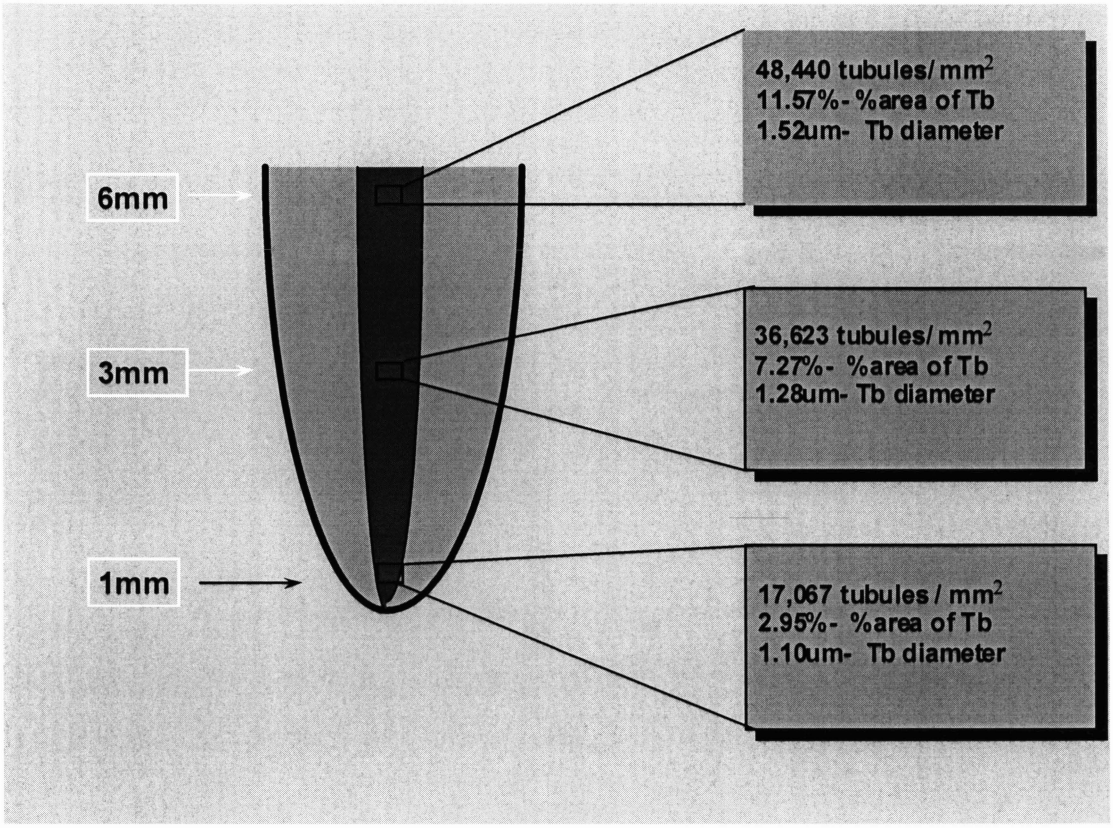


FIG. 23: SUMMARY OF RESULTS.
Mean values calculated at specific sites on the pulpal wall at the apical1mm, 3mm, and 6mm levels.